THE EFFECTS OF REPEATED COCAINE ON STRIATAL GABAERGIC NEUROTRANSMISSION IN SPRAGUE-DAWLEY RATS

By

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THE EFFECTS OF REPEATED COCAINE ON STRIATAL GABAERGIC NEUROTRANSMISSION IN SPRAGUE-DAWLEY RATS

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In humans, repeated cocaine can lead to behavioral sensitization or reverse tolerance. The rat model has been used to study the neurochemical changes associated with the development and maintenance of cocaine sensitization. In rats, cocaine sensitized behavior is measured by increased locomotor activity and stereotypic behaviors. Cocaine is hypothesized to bind to and block the dopamine transporter. Consequently, re-uptake of dopamine is delayed and the dopamine signal is prolonged. However when the effects of repeated cocaine on dopaminergic transmission are examined, these changes cannot solely account for cocaine sensitization. This suggests that other neurotransmitters may be involved in mediating behavioral changes associated with repeated cocaine.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and regulates striatal dopaminergic neurotransmission. $GABA_A$ receptor function and number has been shown to be decreased after cocaine sensitization. Therefore, a decrease in the tonic inhibitory role of striatal GABA could contribute to enhanced striatal dopaminergic output caused by repeated cocaine.

In the experiments described in this dissertation, endogenous GABA release was decreased after cocaine sensitization. The decrease in GABA may be related to the cocaine dose, because only the sensitizing cocaine dose decreased release. Glutamic acid decarboxylase (GAD) activity was not different between repeated saline and cocaine treated rats. In addition, acute *in vitro* cocaine did not affect GAD activity. Next, post-synaptic GABA neurotransmission was examined. Neither allosteric modulation of $GABA_A$ receptor binding nor allosteric modulation of $GABA_A$ receptor-mediated $^{36}Cl^-$ uptake was changed by repeated cocaine. Interestingly, *in vitro* cocaine decreased $GABA_A$ receptor function, and this was not related to repeated cocaine administration. This decrease was restricted to striatal but not cortical $GABA_A$ receptor function. This effect was hypothesized to be caused by protein kinase A phosphorylation of the $GABA_A$ receptor. A cyclic adenosine monophosphate (cAMP) analog, 8-(4-chlorophenylthio)-cAMP sodium, decreased $GABA_A$ receptor function, but the mechanism for this may not be related to $GABA_A$ receptor phosphorylation. Thus, the only measure of striatal GABAergic neurotransmission found to decrease after repeated cocaine was endogenous GABA release. The decline in pre-synaptic GABAergic neurotransmission could enhance the effect of cocaine-stimulated DA neurotransmission and contribute to cocaine sensitization.

CHAPTER 1
LITERATURE REVIEW

History of Cocaine

Cocaine (benzoylmethylecgonine) is derived from the Peruvian coca shrub (*Erythroxylon coca*) (Ellenhorn and Barceloux, 1988). First used medicinally in Western society as a local anesthetic by William Halstead in 1884, the invigorating properties of cocaine led its to inclusion in many consumer health tonics and beverages such as Coca-Cola (Das, 1993). The addictive properties of cocaine were soon realized, and the consumption and distribution of cocaine was regulated by the Harrison Narcotics Act of 1914 (Das, 1993). The abuse of cocaine did not regain national attention until the late 1960s and early 1970s, but cocaine use remained low because of the prohibitively expensive price. With the advent of low-priced "crack" (the sound made when heating chunks of alkaloid cocaine), cocaine use has become a major societal and health problem again (Benowitz, 1993). The 1996 Community Epidemiology Work Group reported that among emergency room visits caused by illicit drugs, cocaine was the most frequently cited in 17 of 20 major U.S. cities, with the number of emergency room visits remaining steady between 1994 and 1995 (NIDA, 1996). Although a number of drugs have been used to treat cocaine-related problems, no effective pharmacotherapy exists for cocaine abusers (Das, 1993; Mendelson and Mello, 1996). Treatment strategies for cocaine abuse and addiction may be dependent on first understanding the direct effects of cocaine on neurons, and then the neuronal adaptations that occur after repeated exposure.

Cocaine Pharmacokinetics and Metabolism

Cocaine is available in two forms, a crystalline salt (cocaine hydrochloride) or the basic form of cocaine. The basic form of cocaine is readily smoked because it is volatile and vaporizes when heated, whereas cocaine hydrochloride decomposes upon heating. The following values were obtained from the review by Ellenhorn and Barceloux (Ellenhorn and Barceloux, 1988). In the blood, cocaine is in its basic form ($pK_A = 8.6$) and readily passes through cell membranes. Cocaine is well absorbed orally (30 – 40% bioavailability) and generally more slowly absorbed through the gastrointestinal and nasal mucosa. Peak human venous blood levels after a typical cocaine dose (0.2 – 2.0 mg kg^{-1}, depending on route) is 200 – 600 ng ml^{-1} (1.9 – 5.8 μM). The time of the concentration peak is highly dependent on the route of administration, with intravenous or smoking being the fastest, which could account for these routes' increased rate of addictiveness. The volume of distribution for cocaine is 1.2 – 1.9 L kg^{-1}.

Cocaine is extensively metabolized by plasma cholinesterase enzymes to mainly two inactive metabolites, ecgonine methylester (32-49%) and benzoylecgonine (29-45%) (Ellenhorn and Barceloux, 1988). Hepatic oxidative metabolism generates the possibly active metabolite norcocaine. Cocaine metabolites are excreted in the urine with less than 10% of cocaine being unmetabolized.

General Cocaine Pharmacology

Cocaine acts both as a local anesthetic agent at high concentrations (> 18 μM) (Przywara and Dambach, 1988; Das, 1993) and as an indirect dopamine (DA) agonist at lower concentrations (< 0.7 μM) by blocking catecholamine and serotonin (5-HT) transporters (Ritz *et al.*, 1987; Woodward *et al.*, 1995; Giros *et al.*, 1996). The local anesthetic effects are due to blockade of the Na^+ channel and the subsequent block of initiation and conduction of electrical impulses in the nerves (Catterall and Mackie, 1996). Cocaine potentiates catecholamine transmission by blocking catecholamine re-uptake, thus prolonging the stimulation of the receptors (Post *et al.*, 1987). The rank order of K_i for cocaine at the following transporters is 5-HT (0.17 μM) > DA (1.51 μM) > norepinephrine (2.93 μM) (Ritz *et al.*., 1987).

Systemic effects of cocaine can result in sympathetic stimulation which can include a rise in acute arterial blood pressure, tachycardia, and seizures (Ellenhorn and Barceloux, 1988). Activation of other adrenergic receptors can also cause mydriasis, hyperglycemia, and hyperthermia (Das, 1993).

In the central nervous system, cocaine acts as a psychomotor stimulant mainly through its binding to the catecholamine transporters, and does not directly stimulate neurotransmitter release, in contrast to amphetamine (Post *et al.*, 1987). In rodents, cocaine binding sites are located throughout the brain, but have the highest concentrations in the striatum and olfactory tubercle (Scatton *et al.*, 1985; Ritz *et al.*, 1990; Shearman *et al.*, 1996). When rats are given a peripheral injection of cocaine, DA levels are elevated in the nucleus accumbens and the striatum (Di Chiara and Imperato, 1988).

Behavioral Effects of Acute, Repeated, and Chronic Cocaine

The behavioral effects of cocaine depend on the frequency of exposure. Upon initial or acute cocaine exposure there can be a range in behavior depending on dose; euphoria, arousal, a sense of vigor and friendliness, anxiety, restlessness, and tremors (Benowitz, 1993). With repeated use of cocaine, tolerance can develop to the euphoric effects and thus stimulate increased dosage after time (Benowitz, 1993). In rats, repeated cocaine causes increased locomotor activity and stereotypic behaviors (Post and Rose, 1976; Ushijima *et al.*, 1994). Stereotypic behaviors include focused sniffing, rearing, repetitive head bobbing and oral movements (chewing, licking, and biting) (see Zahniser and Peris, 1992). Typically, locomotor activity will be displaced by stereotypic behavior because of its focused nature (see Zahniser and Peris, 1992). With repeated exposure to higher dosages of cocaine, rats can exhibit seizures to a formerly subconvulsive dose (Post *et al..*, 1997).

Depending on the delivery schedule of cocaine, rats can either develop sensitization or tolerance. Sensitization is the augmentation in the behavioral effects of cocaine with repeated administration (Hinson and Poulos, 1981). Tolerance to continuous cocaine may result from neurotoxicity and loss of receptors (Ellison, 1992). Intermittent cocaine results in sensitization and is not associated with neurotoxicity (Goodman and Sloviter, 1993). Other stereotypic behaviors, i.e. grooming, head bobbing (headcircling) become enhanced and may even show pattern changes with repeated, intermittent cocaine. The duration of cocaine sensitization after withdrawal from repeated cocaine is also persistent lasting from days (Liu-Chu *et al.*, 1985), to weeks (Post *et al.*, 1987), and months (Henry and White, 1995). Lastly, Pavlovian conditioning can affect the expression of stereotypic behaviors (Hinson and Poulos, 1981).

The metabolites of cocaine have also been shown to cause sensitization. In mice, the administration of repeated cocaethylene in the test environment resulted in increased locomotor activity (Prinssen *et al.*, 1996). However as stated earlier, sensitization can be affected by Pavlovian conditioning (Hinson and Poulos, 1981). Since mice injected in their home cage did not sensitize, the increase in behavior may be a learned response (Prinssen *et al.*, 1996). In another study which eliminated the effects of environmental cues, the administration of repeated cocaethylene in rats resulted in locomotor sensitization (Horger *et al.*, 1996). Since in this dissertation ethanol will not be co-administered with cocaine, it is not likely that the cocaethylene metabolite will be formed and cause sensitization. However, the possibility that cocaine metabolites can cause sensitization should not be completely discounted.

Neuroanatomy of Behavioral Sensitization

Behavioral sensitization or "reverse tolerance" is associated with the mesolimbic and nigrostriatal areas (Koob, 1992; Kalivas *et al.*, 1993). Furthermore, the mesolimbic loop connecting the ventral tegmental area (VTA) and nucleus accumbens (NAc) is hypothesized to be essential in mediating the development of addiction and reinforcement (Kuhar *et al.*, 1991; Koob, 1992). The mesolimbic area is composed of the ventral basal ganglia and DA containing midbrain components (Figure 1-1). The basal ganglia has anatomical links to neuronal areas involved in sensation, movement, cognition and motivation (Wilson, 1990). The neostriatum (striatum; STR) makes up the greatest portion

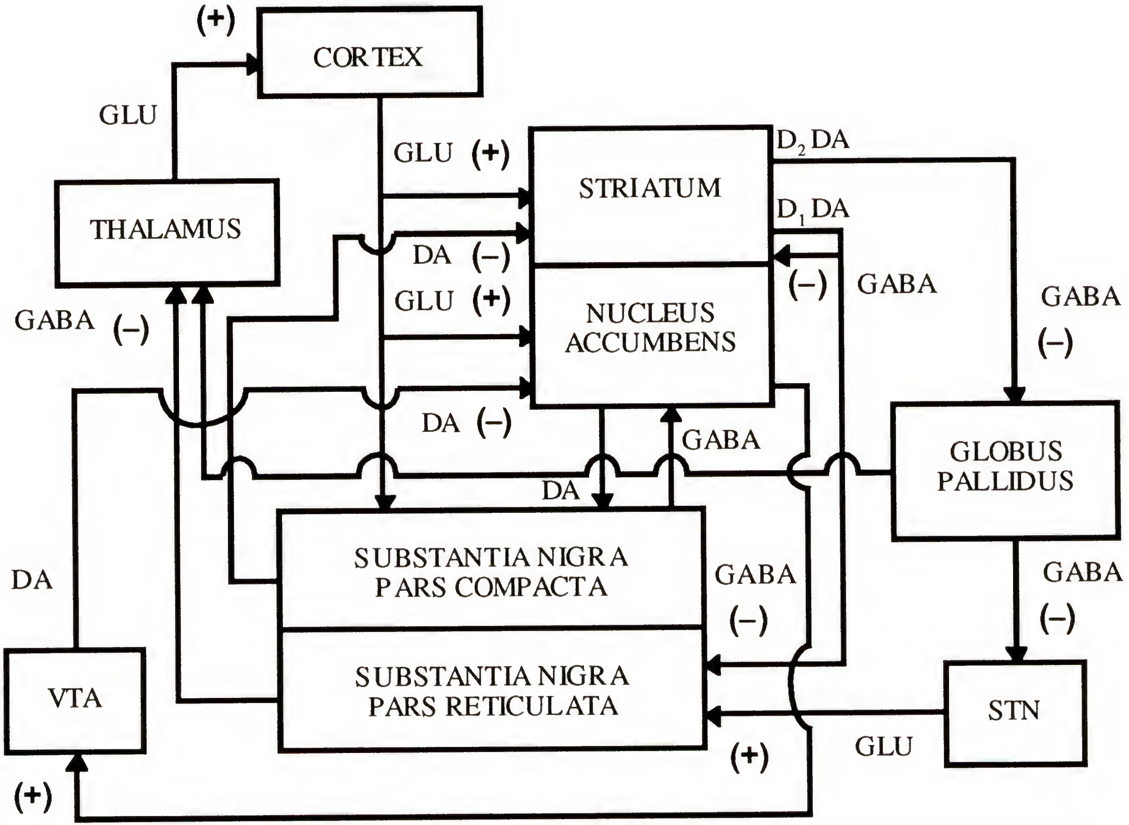

Figure 1-1. Schematic diagram of brain sites and neurotransmitter circuitry in the basal ganglia. STN, subthalamic nuclei; VTA, ventral tegmental area (Gerfen, 1992; Koob, 1992; Schmidt and Kretschmer, 1997).

(in terms of volume) of the basal ganglia and is neuroanatomically comprised of the caudate nucleus, putamen, and NAc (Gerfen, 1988). The remaining components of the basal ganglia are comprised of the globus pallidus (GP), substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr) (Graybiel, 1990). The dopaminergic neurons of the midbrain include neurons of the VTA and retrorubral area (Gerfen, 1992).

The cerebral cortex, SNc and VTA provide inputs to the NAc and STR, and output from the NAc and STR can either go directly to SNc and SNr (striatonigral) or through the GP (striatopallidal). From GP, outputs go to the thalamus or again to SNc and SNr. The STR receives glutamate afferents from the cerebral cortex (corticostriatal) and DA afferents from SNc (nigrostriatal) (Graybiel, 1990). The striatonigral and striatopallidal outputs are GABAergic and inhibitory, but the striatopallidal output disinhibits the subthalamic nucleus and provides a glutamate to the substantia nigra (SN) (Gerfen, 1992). DA cell bodies project from the VTA to the forebrain, largely the NAc, olfactory tubercle, frontal cortex, amygdala and septal areas (Koob, 1992).

The neuronal composition of the striatum is 95% medium spiny neurons (GABA) (Kemp and Powell, 1971; Somogyi and Smith, 1979) and the remainder is of three to six other neuronal types depending on classification (giant aspiny cholinergic neurons (acetylcholine) (aspiny II), medium sparsely spined cells (GABA) (aspiny III), medium smooth and varicose dendrite cells (somatostatin) (aspiny I), and others (Wilson, 1990)). Within the striatum, the medium spiny neurons have extensive arborization and form collateral connections with each other (Gerfen, 1988). Striatonigral neurons are GABAergic and express receptors for both substance P and, dynorphin, as well as the D_1 DA receptor (Gerfen, 1992). Striatopallidal neurons express both enkephalin and D_2 DA receptors (Gerfen, 1992). In the striatum, there is high expression of both D_1 and D_2 DA receptors and low expression of D_3 DA receptors (Meador-Woodruff, 1994).

Other basal ganglia components are the SN and GP. DA cell bodies arise from the SNc, islands of SNr, and retrorubral area (Gerfen, 1992). DA long-axon systems project from the VTA and SN to the striatum; limbic cortex (medial prefrontal, cingulate, and entorhinal areas) (mesocortex); and other limbic structures (the regions of the septum, olfactory tubercle, NAc septi, amygdaloid complex, and piriform cortex) (mesolimbic) (Cooper *et al.*, 1996).

Repeated Cocaine and DA Neurotransmission

In the striatum after repeated cocaine, there is no change in DA transporter (DAT) binding, affinity or number from one day through 60 days after withdrawal (Peris *et al.*, 1990; Pilotte *et al.*, 1994). The ability of the DATs to remove synaptic DA is also not affected by repeated cocaine one or seven days after withdrawal (Peris *et al.*, 1990; Cass *et al.*, 1993). Striatal release of endogenous DA by cocaine is elevated in a dose dependent manner seven days after cocaine withdrawal (King *et al.*, 1993). Amphetamine stimulated striatal [^{3}H]-DA release, is elevated seven days after withdrawal, but not 14 days (Kalivas and Duffy, 1988; Peris *et al.*, 1990). Striatal D_1 and D_2 DA receptors have also been measured after repeated cocaine. Neisewander, Kleven and their colleagues have reported decreased striatal D_1 DA receptor number seven and 14 days after withdrawal, respectively (Kleven *et al.*, 1990; Neisewander *et al.*, 1994). However, Cerruti has reported that there is no change in striatal D_1 DA receptor mRNA 10 days after cocaine withdrawal (Cerruti *et al.*, 1994). D_2 DA receptors show an initial increase immediately after withdrawal (20 min) (Goeders and Kuhar, 1987), followed by no differences in D_2 DA receptor number seven and 14 days after withdrawal (Kleven *et al.*, 1990; Neisewander *et al.*, 1994). It is possible that other changes in striatal dopaminergic neurotransmission may occur after repeated cocaine but were not covered in this review. For example, specific regulation of striatal D_2 DA receptor subtypes or DA receptor coupling to AC may be affected by repeated cocaine, but these have not been specifically examined to my knowledge. In summary, except for changes in stimulated DA release, there are no clear persistent changes in striatal dopaminergic transmission that could account for enhanced sterotypy with repeated cocaine at all time points at which the behavior occurs.

The neurochemical changes in the NAc after cocaine withdrawal have been recently reviewed (Kuhar and Pilotte, 1996). After cocaine withdrawal, the ability to terminate DA neurotransmission in the NAc remains persistently impaired. There is a persistent decrease in DATs 10 days after cocaine withdrawal (Pilotte *et al.*, 1994). The function of the remaining DATs is also impaired, so that the clearance of DA from the synaptic area is significantly reduced (Cass *et al.*, 1993). There is, however, some neuronal compensation, so that there is less basal DA release during the development of cocaine sensitization and as long as six days after withdrawal (Hurd *et al.*, 1989; Imperato *et al.*, 1992). Others have seen cocaine-stimulated increases of DA release in the NAc during or after intermittent cocaine (Kalivas and Duffy, 1990; Ng *et al.*, 1991).

With respect to the number of D_1 DA receptors in the NAc after cocaine withdrawal, there are reports of no change after one day (Peris *et al.*, 1990), decreases after seven and 14 days (Neisewander *et al.*, 1994), no change and decreases after 30 days (Henry and White, 1991; Neisewander *et al.*, 1994) and finally no differences after 60 days (Neisewander *et al.*, 1994). Functionally, post-synaptic D_1 DA receptors in NAc are more sensitive for up to one month after repeated cocaine when tested electrophysiologically (Henry and White, 1991), although this increased sensitivity is not due to an increase in D_1 DA receptor binding or activation of adenylate cyclase (AC) (Mayfield *et al.*, 1992). D_2 DA receptors in the NAc show initial increases one day after withdrawal (Peris *et al.*, 1990), followed by no change after seven or 14 days (Kleven *et al.*, 1990; Peris *et al.*, 1990; Neisewander *et al.*, 1994). The sensitivity of pre-synaptic D_2 DA receptors can also be decreased or increased by continuous or intermittent cocaine (Dwoskin *et al.*, 1988; Henry *et al.*, 1989; King *et al.*, 1994). In summary, changes in DA receptors or their G-coupled proteins do not seem to be the sole source in the development and maintenance of behavioral sensitization since the time course of these changes does not correlate with the time course of behavioral changes. Thus, some other neurotransmitter must contribute to the development and maintenance of cocaine sensitization. Other neurotransmitters implicated in sensitization include, γ-aminobutyric acid (GABA), glutamate and norepinephrine

GABA Neurotransmission

GABA is the major inhibitory neurotransmitter in the brain. GABA is enzymatically synthesized by glutamic acid decarboxylase (GAD) through the α-decarboxylation of glutamate. GABA is metabolized in succession by GABA-trans-aminase (GABA-T) and succinic semialdehyde dehydrogenase into succinic acid (Cooper *et al.*, 1996). By means of the "GABA shunt," the breakdown of GABA allows for the indirect recycling of GABA back to glutamate, which is the substrate for GAD (Cooper *et al.*, 1996). The "GABA shunt" involves the energy created in the breakdown of GABA by GABA-T for the generation of glutamate from α-ketogluterate. In addition, an amine group is conserved. GABA is a unique neurotransmitter because it is a electroneutral zwitterion (pI = 7.3) at physiological pH, which allows for low impediment when traveling across the synaptic cleft (Roberts and Sherman, 1993).

In 1950, Awapara and Roberts independently first isolated GABA from the brains of rats and mice (Awapara *et al.*, 1950; Roberts and Frankel, 1950). GABA was then characterized as a neurotransmitter in crustacean neuromuscular junctions by 1963. Not until the 1980s, did description by electrophysiological, biochemical, anatomical, and behavioral experiments allow GABA to fulfill the major criteria for neurotransmitter action. The review by Rabow summarizes the major evidence arguing for classification of GABA as a neurotransmitter in spine neuronal cell culture (Rabow *et al.*, 1995). GABA production was localized to brain nerve endings by sucrose gradient centrifugation (Weinstein *et al.*, 1963) and to spinal cord neurons by GAD immunocytochemical labeling (McLaughlin *et al.*, 1975). In cat studies, application of GABA caused a negative shift in membrane potential similar to endogenous inhibitory synaptic potentials (Krnjevic and Schwartz, 1967; Dreifuss *et al.*, 1969). Application of GABA to spinal neurons caused a response similar to electrophysiological changes caused by endogenous inhibitory synaptic potentials (Curtis *et al.*, 1968; Barker and Nicoll, 1973). GABA release was stimulated by both electrical current and high K^+ concentrations (Srinivasan *et al.*, 1969) and also was Ca^{2+} dependent (Levy *et al.*, 1973). Cerebral cortex slices were shown to inactivate GABA by rapid uptake of GABA (Iversen and Neal, 1968). In spinal neuronal cultures, $[^3H]$-GABA was demonstrated to be taken up, stored, and released by K^+ depolarization in a Ca^{2+} dependent manner (Farb *et al.*, 1979). Thus, GABA meets the criteria of neurotransmitter action: synthesis, storage, release, post-synaptic action and inactivation.

GABA Receptors

GABA directly affects two neuronal receptor types: $GABA_A$ receptors, which are coupled to a Cl^- channel; and $GABA_B$ receptors, a G-protein linked receptor. $GABA_A$ receptors serve as inhibitory channels because when stimulated they allow Cl^- ions into the cell, which tends to repolarize or hyperpolarize the cell. $GABA_B$ receptors are a G-protein coupled receptor (Bowery, 1989; Bettler *et al.*, 1998). Although $GABA_B$ receptors do not directly pass ions through a channel in the receptor, it is capable of decreasing the conductance of Ca^{2+} and K^+ through actions on other ion channels (Bowery, 1989). Clinically, the $GABA_A$ receptor is the more important receptor because it can be modulated by benzodiazepines (BZDs), barbiturates, steroid hormones, ethanol and ß-carbolines (Sieghart, 1995). More recently, however, there is some evidence for the importance of the $GABA_B$ receptor in the prevention of drug abuse (Ashby *et al.*, 1999). $GABA_B$ receptors were most recently reviewed by Bettler and colleagues (Bettler *et al.*, 1998). The relationship between $GABA_B$ receptors and cocaine sensitization will not be addressed further because the pharmacological tools for manipulating $GABA_B$ receptors are limited and $GABA_B$ receptors are not the primary focus of this dissertation.

There are many excellent reviews on the $GABA_A$ receptor, with the most thorough and current accounts being by Sieghart (Sieghart, 1995) and Rabow (Rabow *et al.*, 1995). $GABA_A$ receptors belong to the superfamily of ligand gated ion channels and much of what is known about the receptor was first identified based on the structure and function of the nicotinic acetylcholine receptor. The molecular structure of the $GABA_A$ receptor is thought to be a heteropentameric glycoprotein of about 275 kDa (DeLorey and Olsen, 1992). The $GABA_A$ receptor is composed of distinct polypetide subunits that have been identified and classified as subtypes: α(1-6) (Schofield *et al.*, 1987; Levitan *et al.*, 1988; Khrestchatisky *et al.*, 1989; Ymer *et al.*, 1989a; Kato, 1990; Malherbe *et al.*, 1990), ß(1-4) (Schofield *et al.*, 1987; Ymer *et al.*, 1989b; Bateson *et al.*, 1991), γ(1-4) (Pritchett *et al.*, 1989; Ymer *et al.*, 1990; Wilson-Shaw *et al.*, 1991; Harvey *et al.*, 1993), δ (Shivers *et al.*, 1989) and ρ(1-2) (Cutting *et al.*, 1991; Cutting *et al.*, 1992). Additionally, some subunits such as γ_2 have splice variants that have individual differences (Kofuji *et al.*, 1991). The subunits of a family share 70 – 80% amino acid homology, while the different families share only 30 – 40% between them (Seeburg *et al.*, 1990). The subunits are similar in size, 450 – 550 amino acids in length and between 53 – 57 kDa (Seeburg *et al.*, 1990; DeLorey and Olsen, 1992). The cDNA encodes for a subunit that has a large NH_2-terminal extracellular domain, four putative α-helical hydrophobic membrane spanning regions and a small extracellular COOH terminus (Seeburg *et al.*, 1990). The NH_2-terminal extracellular domain contains multiple N-linked glycosylation sites and Cys residues important for forming a ß-structural loop needed to bind GABA. The second transmembrane spanning region (M2) contains a conserved octapeptide sequence containing several hydrophilic residues proposed to line the pore of the $GABA_A$ receptor analogous to the nicotinic acetylcholine receptor (reviewed by Seeburg *et al.*, 1990). Between transmembrane spanning region three and four (M3-M4) exists a large, poorly conserved cytoplasmic loop that often contains putative phosphorylation sites for PKA and PKC (Schofield *et al.*, 1987).

Although GABA$_A$ receptor subunit composition and stoichiometry *in vitro* is unknown, the significance of the individual contribution of a subunit to receptor function has been partially resolved. α Subunits contain the BZD binding site with the α_1 subunit having the highest affinity for BZD. Two sites on the α subunit are critical for BZD binding and allosteric modulation of GABA$_A$ receptor function, Gly201 and His102 of α_1 (Pritchett and Seeburg, 1991; Wieland *et al.*, 1992). ß Subunits are required for successful expression of the GABA$_A$ receptor (Wisden *et al.*, 1992b; Connolly *et al.*, 1996) and also affect GABA affinity (Bureau and Olsen, 1990). BZD binding occurs on the α subunit, but functional BZD sensitivity does not occur unless a γ subunit is also present (Verdoorn *et al.*, 1990). The δ subunit replaces the γ subunit and makes the GABA$_A$ receptor insensitive to BZDs (Shivers *et al.*, 1989). ρ Subunits have only been identified in the retina and will not be discussed further. Thus, GABA$_A$ receptor function can be affected by the subunits comprising the receptor.

The various combinations of different subunits can create a large number of potential GABA$_A$ receptors with one early estimate being approximately 152,000 types for 15 subunit types, which does not include subunit splice variants (Burt and Kamatchi, 1991). It appears, however, that the variety of GABA$_A$ receptors that actually co-assemble is smaller. When the distribution of neuronal GABA$_A$ receptor subunit mRNA was examined by Wisden and colleagues, they concluded that some subunits preferentially would combine and form an active receptor, e.g. $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_x$, $\alpha_5\beta_1\gamma_x$, where x represents any of the various γ-variants (Wisden *et al.*, 1992b). The combination of $\alpha_1\beta_2\gamma_2$ seems to have the greatest distribution and its electrophysiological function matches "classical" GABA$_A$ receptor function in co-expression experiments (Sigel *et al.*, 1990; Verdoorn *et al.*, 1990). The distribution of GABA$_A$ receptor subunits within the striatum is mainly α_2, α_4, β_3, γ_2 and γ_3, with the other subunits having low expression (Wisden *et al.*, 1992a), which may be critical to the development of cocaine sensitization (Resnick *et al.*, 1999). One of the limitations of studies involving $GABA_A$ receptor function or binding is that neither the subunit composition of the receptor, nor stoichiometry is precisely known. Eventually, this limitation must be overcome because identifiable differences have been observed between $GABA_A$ receptors located on the soma and the dendrites of the same rat pyramidal neuron (Alger and Nicoll, 1982). The ability to identify receptor subunit composition and stoichiometry will allow for distinction between subunit heterogeneity and other types of receptor modification.

Modulation of $GABA_A$ Receptor Function

Like all receptors, the regulation of $GABA_A$ receptor function can be affected by a wide variety of mechanisms. Typical changes can be classified into at least two types: pharmacological and structural. Pharmacologically, the $GABA_A$ receptor is known to be directly modulated by BZDs, barbiturates, ß-carbolines and ethanol (DeLorey and Olsen, 1992). Structurally, receptor subunit composition has already been demonstrated to play an important role. Additionally, other structural mechanisms that can change receptor function are the amount of active receptors expressed (DeLorey and Olsen, 1992) and modification by phosphorylation of the receptor (Moss *et al.*, 1992a; Moss *et al.*, 1992b). The number of active receptors expressed can be determined, by RNA levels, mRNA stability, protein expression, and protein degradation (Rabow *et al.*, 1995). These changes are very dramatic and can show an “all-or-none” effect (Rabow *et al.*, 1995). The more subtle structural change is modification by phosphorylation of the receptor. This is more subtle because depending on the number of phosphorylation sites on the receptor, the effect can be in a range between the “all-or-none” effect (Krishek *et al.*, 1994). Phosphorylation of the $GABA_A$ receptor will be discussed at length below.

Pharmacologic Modification of GABA$_A$ Receptor Function

Direct agonists, such as GABA and muscimol, bind to the GABA binding site, where binding of either ligand will cause receptor activation. Bicuculline is a competitive antagonist that also binds to this site and inhibits receptor function (Olsen and Snowman, 1983). In addition to the GABA binding site, the GABA$_A$ receptor has multiple ligand binding sites, which can increase or decrease receptor function. These binding sites are known as non-competitive binding sites because the modulation ligand does not compete or act on the same site as does GABA. Some examples of indirect agonists mentioned before are BZDs, barbiturates, steroid hormones, ethanol and ß-carbolines (Sieghart, 1995).

Structural Modification of GABA$_A$ Receptor Function by Phosphorylation

Phosphorylation of the GABA$_A$ receptor can affect the function of the ionophore (Moss *et al.*, 1992a; Moss *et al.*, 1992b). When examined over time, phosphorylation of a receptor has an intermediate effect upon receptor function. Direct and indirect agonists have an immediate effect on receptor function, whereas protein expression and degradation regulate receptor function over a longer time period (Rabow *et al.*, 1995). The effect of phosphorylation can also be short term or long term because a phosphorylated protein can be dephosphorylated. Since modulation of receptor phosphorylation can lead to such diverse responses, it is not surprising that there are several ways to phosphorylate a receptor. Two pathways will be discussed in detail below: protein kinase A (PKA) and protein kinase C (PKC) pathways

PKA activation is the result of the activation of G_s by an external signal. When G_s is stimulated it activates adenylyl cyclase (AC), which catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP can then activate PKA (Cooper *et al.*, 1996). The second pathway is PKC. When G_0 is activated by an external signal, the enzyme phospholipase C (PLC) is activated. PLC cleaves PIP_3 to yield diacylglycerol (DAG) and

IP_3. DAG allows the activation of PKC, while IP_3 increases intracellular calcium, which activates CaM kinase II (CaM KII), another enzyme capable of kinase activity (Cooper *et al.*, 1996; Csukai and Mochly-Rosen, 1999). The PKC isozymes and their localization was reviewed by Csukai and Mochly-Rosen (Csukai and Mochly-Rosen, 1999).

Protein Kinase A

The modulation of certain $GABA_A$ receptor subunits by phosphorylation may be essential in affecting the receptor function. Heuschneider and Schwartz found that they could decrease chloride flux stimulated by muscimol in rat synaptoneurosomes when cAMP analogs or forskolin were used (Heuschneider and Schwartz, 1989). Forskolin was used to induce AC activity and showed an inhibitory dose response curve for chloride uptake. cAMP levels were also measured and paralleled the response curve. This provided strong evidence of a possible role of PKA to inhibit $GABA_A$ receptor function. It was also demonstrated in a more detailed study that cAMP analogs decreased $GABA_A$ receptor $^{36}Cl^-$ uptake and this increase was associated with a increase in PKA activity (Schwartz *et al.*, 1991). Thus, phosphorylation of the $GABA_A$ receptor by PKA second messenger pathways regulates $GABA_A$ receptor function.

Further evidence for $GABA_A$ receptor desensitization was shown in chick cortical neurons (Tehrani *et al.*, 1989). cAMP and several stable analogs increased the rate at which $GABA_A$ receptors desensitized when measuring chloride uptake. The actions of cAMP should be viewed with caution. Even though application of cAMP to neuronal cells decreased cellular inward current response by GABA, the response was not due to phosphorylation of the receptor, but rather the effect of external application of cAMP or analogs (Harrison and Lambert, 1989; Lambert and Harrison, 1990). In addition, the effect of cAMP analogs had a phosphorylation independent effect on GABA-stimulated $^{36}Cl^-$ uptake (Leidenheimer *et al.*, 1990; Leidenheimer *et al.*, 1991). Thus, when determining the effects of cAMP analogs, the phosphorylation of the $GABA_A$ receptor must also be determined.

Molecular methods using reconstituted $GABA_A$ receptors also confirmed that PKA was capable of modulating $GABA_A$ receptor activity. Moss and colleagues transiently transfected the $GABA_A$ receptor subunits into embryonic kidney cells (Moss *et al.*, 1992b). When cellular cAMP was induced by forskolin, chloride inward currents were reduced as measured by whole cell patch clamping. When the cells were analyzed by immunocytochemistry, the $ß_1$ subunit was identified as the phosphorylated target. Chloride current was also reduced by phosphorylation of the $ß_1$ subunit, but the possible effects caused by $GABA_B$ receptor stimulation were not eliminated. In addition, they could not detect whether the γ_2 subunit was phosphorylated because it could not be isolated (Moss *et al.*, 1992b). In contrast, $ß_1$ subunit phosphorylation has also been shown to enhance $GABA_A$ receptor function (Angelotti *et al.*, 1993). This group showed that $GABA_A$ receptors ($\alpha_1ß_1\gamma_2$) transiently expressed in a mouse fibroblast cells having different levels of PKA activity had enhanced GABA current, which was attributable to the $ß_1$ subunit. Currently, these laboratories do not have a reasonable explanation for their differences (Kapur and MacDonald, 1996; McDonald *et al.*, 1998). Although the effect of PKA phosphorylation is not clear, phosphorylation of the $GABA_A$ receptor has been definitely established (Tehrani and Barnes, 1994). Thus, the standard for examining the effects of PKA phosphorylation of the $GABA_A$ receptor requires both the status of phosphorylation and the physiologic function of the $GABA_A$ receptor.

PKA has specific target sites on $GABA_A$ receptor subunits for phosphorylation. First demonstrated by Kirkness and colleagues in isolated $GABA_A$ receptors from porcine cortex and soon followed by Browning and colleagues, it was shown that specific subunits were targets of phosphorylation by PKA (Kirkness *et al.*, 1989; Browning *et al.*, 1990). Specifically, cAMP-dependent protein kinases phosphorylated a ß subunit (55 kDa, now $ß_1$), but not two α subunits. PKC was not detectable in this system, which could be due to the receptor isolation technique which included antagonist to PKC enzymes (Kirkness *et al.*, 1989). Purified PKA can phosphorylate a ß subunit (58 kDa, now ß$_3$) in GABA$_A$ receptors isolated from bovine brain (Browning *et al.*, 1990). More direct evidence for phosphorylation of GABA$_A$ receptor subunits was shown when the ß$_1$ PKA phosphorylation site was changed from Ser409 to Ala (Moss *et al.*, 1992a). This change resulted in the elimination of the GABA response to cAMP and suggests a physiological role for the phosphorylation of the ß$_1$ subunit by PKA. More recently, it was found that the ß$_3$ subunit contains two PKA phosphorylation sites (S408 and S409) that when phosphorylated increased GABA$_A$ receptor function (McDonald *et al.*, 1998), which was in contrast to the decrease seen with the phosphorylation of the ß$_1$ subunit (Moss *et al.*, 1992a). However, phosphorylation of the ß$_2$ subunits PKA site did not change GABA$_A$ receptor function. Differences in cellular response to PKA were demonstrated between pyramidal neurons and dentate gyrus granule cells (Poisbeau *et al.*, 1999), which may be due to these GABA$_A$ receptor subunit differences. Thus, the regulation of GABA$_A$ receptor function by PKA phosphorylation may be determined by GABA$_A$ receptor subunit expression.

Protein Kinase C

The effects of PKC induced activity on ion channels and the GABA$_A$ receptor were measured in oocytes injected with chick forebrain mRNA for GABA$_A$ receptor subunits (Sigel and Baur, 1988). The phorbol ester, 4ß-phorbol 12-myristate 13-acetate (PMA) was used to activate PKC activity in these transfected cells. A reduction in GABA stimulated current was observed when PMA was pre-incubated with the cells. PMA activation of PKC activity was blocked by the addition of tamoxifen, a PKC inhibitor. These results provided strong evidence for a role for PKC activity in modulating GABA$_A$ receptor function.

In another study, PKC activity had a role in GABA$_A$ receptor desensitization that occurred at a specific state during receptor activation (Leidenheimer *et al.*, 1992). Specifically, PMA worked in a dose dependent manner, while the inactive phorbol ester did not have an effect. Using resealed microsacs where protein phosphorylation was inhibited, the authors showed that PMA activity was blocked. Thus, the specific phosphorylation of the $GABA_A$ receptor by PKC inactivated the receptor. The time course of uptake was examined and showed a decrease during the slow phase of uptake in the presence of PMA. Lastly, Leidenheimer and colleagues showed that by inhibiting PKC activity, receptor function was spared in the presence of PKC, but other phosphatase inhibitors did not show this protective effect. Again, this study provided strong evidence for a role for both PKC activity and phosphorylation to regulate $GABA_A$ receptor function.

Site-directed mutagenesis experiments show that the $GABA_A$ receptor is regulated by PKC phosphorylation in two cellular systems (Krishek *et al.*, 1994). First, they demonstrated the ability of PKC to phosphorylate serine residues on the $ß_1$ subunit in a cellular system. When functional tests were performed on these transiently transfected cells in the presence of PDBu, a PKC activator, there were only slight changes in response and phosphorylation. In order to study this modest response, the amount of inducible PKC activity was overexpressed by transfecting their cells with PKC cDNA. This change dramatically affected the results such that a 45% reduction in peak amplitude was now apparent. The authors showed a decreased response when the γ_{2L} subunit was present compared to the γ_{2S}. The authors next changed the target amino acid of phosphorylation for PKC in the $ß_1$(S409) and γ_{2L}(S327/S343) subunits. When the function of these modified subunits was studied, conductance was unchanged when compared to controls, while the unmodified receptors showed decreased activity. From equilibrium concentration-response relationships, it was determined that the number of phosphorylation sites determined the degree of inhibition of the current.

The decrease in $GABA_A$ receptor function caused by PKC may also be related to the down regulation of the receptor. When the effects of PKC activation on $GABA_A$ receptors in oocytes was examined, both a decrease in GABA current and a 65% decrease in cell surface expression of $GABA_A$ receptors were evident (Chapell *et al.*, 1998). These decreases were found to be specific for the PKC phosphorylation site on the GABA$_A$ receptor. Thus, PKC decreases both GABA$_A$ receptor function and number.

When phosphorylation of the isolated GABA$_A$ receptor by PKC was assessed, the ß$_1$ subunit (56 kDa) was found to be phosphorylated by the kinase (Browning *et al.*, 1990). This subunit has a distinct pattern separate from ß$_3$ (58 kDa) which was phosphorylated by PKA. Additional sites for PKC phosphorylation were identified on the splice variant of the γ_{2L} subunit contained on the additional length of the protein in the intracellular loop between the third and fourth transmembrane proteins (Whiting *et al.*, 1990). In the oocyte expression system, the ß$_2$(S410) and γ_{2S}(S327) subunits of the GABA$_A$ receptor contain phosphorylation sites that reduce inward current in the presence of PMA (Kellenberger *et al.*, 1992). In these experiments, the individual replacement of γ_{2S} (S327A) reduced current amplitude the most, but when the ß$_2$ (S410A) site was also changed the individual effects were not additive.

Krishek and colleagues also described in their set of experiments the relative importance of each individual PKC phosphorylation site in the GABA$_A$ receptor subunits (Krishek *et al.*, 1994). In the presence of the γ_{2L} subunit, the phosphorylation of the ß$_1$ site did not provide additional inhibition. However, when the two sites of phosphorylation on γ_{2L} were considered individually and together, the site within the sequence unique to the γ_{2L} contributed more to current reduction than the site commonly found on both γ_2 subunits. The inhibitory action of phosphorylation seems saturable since there was no additional effects when all three sites were present in the receptor.

The regulation of individual PKC isozymes is just beginning to be understood because the pharmacologic tools to examine isozyme specific roles have recently been made available (Csukai and Mochly-Rosen, 1999). The regulation of the GABA$_A$ receptor by these specific PKC isozymes will be of extreme interest.

Other Protein Kinases

There are other possible sites of regulation on the GABA$_A$ receptor by protein phosphorylation. CaM kinase II can phosphorylate the additional eight amino acid peptide sequence found in the γ_{2L} subunit, but not in γ_{2S} (Machu *et al.*, 1993). It is possible that through the shared homology of the sequence necessary for identification by the PKC and CaM kinase II that both kinases can regulate receptors containing this subunit. Additional evidence for phosphorylation of the γ_2 site was inferred from the phosphorylation of the ß$_1$ subunit (McDonald and Moss, 1994). These authors used site directed mutagenesis to determine the amount of phosphorylation by CaM kinase II and cGMP-dependent protein kinase on previously known targeting sites of the receptor (the intracellular loop between transmembrane protein 3 and 4). The sites known to be targeted by CaM kinase II were ß$_1$ (S409 and S384) and γ_{2S} and γ_{2L} (both S348 and T350, S343 γ_{2L} additionally). Currently, there are no functional studies to determine whether CaM kinase II has a direct role in regulating GABA$_A$ receptor function like both PKA and PKC does.

Moss and colleagues described a tyrosine phosphorylation site which increased conductance when GABA was applied (Moss *et al.*, 1995). They artificially increased kinase activity by increasing tyrosine kinase levels. In this experiment, they showed that specific tyrosine residues on the ß$_1$ and γ_{2L} subunits were phosphorylated. When functional studies were performed, GABA stimulated current increases. However when tyrosine sites were removed or tyrosine kinase inhibitors were added, GABA stimulated conductance was decreased.

The last protein kinase to affect GABA receptor function is more mysterious. Sweetnam and colleagues observed that the α subunit was phosphorylated on a serine residue (Sweetnam *et al.*, 1988). However when they used various inhibitors of protein kinases and second messengers, cAMP, and Ca^{2+}, they could not affect the phosphorylation of the subunit. This unknown kinase has been since known as the "unidentified, receptor-associated protein kinase."

GABA Modulation of DA Neurotransmission

GABAergic and dopaminergic neurons in the striatum form circuits that balance and oppose the other's actions (see Figure 1-1, pg. 5). Striatal DA release results in a net excitatory effect on locomotion via the striatonigral pathway and a net inhibitory effect on movement via the striatopallidal pathway (Sommer *et al.*, 1996). GABA neurotransmission regulates and inhibits striatal dopaminergic transmission (Reid *et al.*, 1990a; Reid *et al.*, 1990b; Gruen *et al.*, 1992; Ronken *et al.*, 1993; Smolders *et al.*, 1995; Dewey *et al.*, 1997). Thus, it is possible that a decrease in GABAergic neurotransmission caused by repeated cocaine would enhance striatal dopaminergic output and explain behavioral sensitization (Pecins-Thompson and Peris, 1993; Peris, 1996). There is no evidence, however, for the direct modulation by cocaine of the $GABA_A$ receptor.

Cocaine could indirectly decrease GABAergic neurotransmission through dopaminergic activation of signal transduction pathways. As described earlier, $GABA_A$ receptor function is highly regulated via PKA and PKC-dependent phosphorylation events. Repeated cocaine increases AC and PKA activity in the NAc and is speculated to facilitate the acquisition of locomotor sensitization and/or drug addiction (Terwilliger *et al.*, 1991; Miserendino and Nestler, 1995; Nestler, 1997). Although similar increases in AC and PKA activity were not found in the striatum, more important may be changes in the regulation of AC or PKA after repeated cocaine, which was not measured in these experiments (Terwilliger *et al.*, 1991). Thus, a decrease in the tonic inhibitory role of striatal GABA could contribute to enhanced striatal dopaminergic output caused by repeated cocaine.

Acute and Repeated Cocaine on Pre-synaptic GABA Neurotransmission

Cocaine can have its effect on GABAergic neurotransmission pre-synaptically, post-synaptically or both. The majority of previously reported literature has concentrated on post-synaptic effects of cocaine, but the data suggest that cocaine does affect pre-synaptic GABA neurotransmission. The limited work done on pre-synaptic mechanisms have focused on GABA production and release.

GABA production can be regulated by its synthetic enzyme GAD. Initially, GAD activity was found to be increased in cocaine treated rats (Gale *et al.*, 1981). More recently, GAD mRNA was found to be decreased in the NAc after repeated cocaine treatment (Sorg *et al.*, 1995). It has also been reported that there was a decrease in cocaine induced seizure threshold after GAD antisense treatment in mice (Abel and Kirages, 1995). However, cocaine treated rats that were behaviorally sensitized did not show a change in the number of GABA-positive amygdala neurons (Lee *et al.*, 1991). In another experiment, rats self administering cocaine or rats yoked to the self administering cocaine rats did not change GABA content in either the NAc or STR (Dworkin *et al.*, 1995). These authors also showed that turnover of GABA was increased in the STR and SN of the cocaine self-administering rats (Dworkin *et al.*, 1995). These data suggest that the most important change caused by repeated cocaine may be the rate at which GAD produces GABA, although the absolute amount of GAD may also be important in determining the amount of GABA produced.

In vitro cocaine at local anesthetic concentrations (0.2 – 1.0 mM) decreases GABA release from rat whole brain synaptosomes without altering GABA synthesis or breakdown (Ikeda *et al.*, 1983). Rats given systemic *in vivo* cocaine (15 mg kg^{-1} i.p.) also show a decrease in VTA GABA release, and this effect was not blocked by a D_1 DA antagonist (Kalivas, 1995). However, VTA glutamate release was increased by the same treatment of cocaine and was blocked by the D_1 DA antagonist (Kalivas *et al.*, 1995). In the dorsolateral septal nucleus (DLSN), GABA release was increased after repeated cocaine (Shoji *et al.*, 1997). The authors found that DLSN pre-synaptic $GABA_B$ autoreceptors have diminished effectiveness which leads to increased GABA release after repeated cocaine for 14 or 28 days, but not seven days (Shoji *et al.*, 1997). In a related model, chronic cocaine (indwelling cocaine pellet over five days) did not alter GABA release into the STR (Keys and Ellison, 1994), but continuous cocaine is not representative of typical cocaine abuse patterns and also induces neurotoxicity.

Acute and Repeated Cocaine on Post-synaptic GABA Neurotransmission

The effects of *in vitro* cocaine have been measured on post-synaptic currents, mainly to study cocaine's convulsant and anesthetic effects. In the hippocampus, cocaine did not have a selective depressant effect on local inhibitory circuitry in CA1 pyramidal neurons (Dunwiddie *et al.*, 1988). Although cocaine did not directly affect $GABA_A$ receptor function, cocaine (100 μM) did affect both $GABA_A$ and $GABA_B$ receptor mediated inhibitory post-synaptic potentials (Dunwiddie *et al.*, 1988). In a more recent study, cocaine (127 μM and 1.13 mM) was shown to decrease hippocampal GABA current after pre-incubation and acute application (Ye *et al.*, 1997).

In this lab and others, it has been demonstrated that the number of $GABA_A$ receptor convulsant binding sites in the striatum of sensitized rats is reduced after cocaine withdrawal (Gale *et al.*, 1981; Pecins-Thompson and Peris, 1993). Others have indicated that there is an increase in the BZD binding sites after repeated cocaine in the rat model (Goeders *et al.*, 1990; Goeders, 1991), but these increases are reversed after 14 days of withdrawal. More recently, when BZD binding was examined by the same group in a task reward model with cocaine being injected before or after the task completion, the VTA was the only site measured that had a decrease in binding in rats treated with cocaine (Goeder *et al.*, 1997). Data for both NAc or STR were not shown (Goeders *et al.*, 1997). In rats treated continuously with an indwelling cocaine pellet for five days, BZD binding was increased in the lateral and medial caudate 30 days after withdrawal of chronic cocaine (Zeigler *et al.*, 1991). This group also showed that five days of chronic cocaine was necessary to induce an increase in striatal BZD binding and that there was increased binding both 12 hr and 21 days after the withdrawal, but not two days (Lipton *et al.*, 1995). Although these studies are highly informative, the binding assays did not measure the functional ability of the bound $GABA_A$ receptors. If *in vitro* , the $GABA_A$ receptors can bind ligand, but *in vivo*, they are not functional, then the increases in $GABA_A$ receptor number by some of the groups would be less meaningful.

Summary

The neurochemical mechanism underlying cocaine sensitization after repeated or intermittent cocaine remains unclear. Although DA release is increased after cocaine sensitization, this change alone cannot account for behavioral sensitization. Inhibitory GABA neurotransmission regulates and inhibits striatal dopaminergic transmission. Thus, it is possible that decreases in GABAergic neurotransmission caused by repeated cocaine would enhance striatal dopaminergic output and explain behavioral sensitization (Pecins-Thompson and Peris, 1993; Peris, 1996). It has already been shown that there are both pre- and post-synaptic changes in striatal GABAergic neurotransmission after cocaine sensitization. However, some of the identified changes are equivocal. Therefore, the studies presented in this dissertation were designed to enhance the understanding of the changes in striatal GABAergic neurotransmission after cocaine sensitization.

Objective

The general hypothesis of this dissertation was that decreased striatal GABAergic neurotransmission contributes to the development, expression and maintenance of cocaine sensitized behavior. Since this hypothesis encompasses a wide range of changes caused during cocaine sensitization, the primary motive was to identify specific striatal GABAergic changes after cocaine sensitization. The rationale was that significant changes in striatal GABAergic neurotransmission after cocaine sensitization are critical for at least the maintenance and expression of sensitization. The next step was then to identify the mechanism behind the observed change. The experiments were designed to test three separate areas where repeated cocaine could affect striatal GABAergic neurotransmission.

First, the effects of repeated cocaine on pre-synaptic striatal GABA release were examined. Previously, pre-synaptic striatal GABAergic changes were not examined even though post-synaptic changes had been identified in cocaine sensitized rats. Thus, it was important to determine if these post-synaptic changes were a compensatory response to pre-synaptic changes caused by repeated cocaine. In the designed studies, endogenous and exogenous GABA were measured from the striata of rats behaviorally sensitized to cocaine (Chapter 3). Striatal GAD activity was measured to test possible mechanisms behind the decreases observed in striatal endogenous GABA (Chapter 4).

Second, the effects of repeated cocaine on post-synaptic striatal $GABA_A$ receptor binding was determined. The studies to date are equivocal and difficult to compare. In addition, the experiments measured different binding sites on the striatal $GABA_A$ receptor and did not test the functionality of these receptors. For the experiments in this dissertation, the endogenous ligand (muscimol) and BZD binding sites were examined after cocaine sensitization. In addition, the ability of these binding sites to be altered functionally by a second $GABA_A$ receptor ligand was measured (Chapter 5).

Third, striatal $GABA_A$ receptor function was tested after *in vitro* and *in vivo* cocaine. The effects of cocaine can have both an acute and persistent effect on striatal $GABA_A$ receptor function. Acute exposure of cocaine could have a direct or indirect effect on striatal $GABA_A$ receptor function. Persistent exposure to *in vivo* cocaine could change striatal $GABA_A$ receptor function by either affecting receptor subunit composition or the ability of the receptor to be modulated by allosteric modulators. In this dissertation, the function of the striatal $GABA_A$ receptor was examined in two parts. First, the modulation of striatal $GABA_A$ receptor function was examined after *in vivo* cocaine (Chapter 6). Secondly, striatal $GABA_A$ receptor function was examined after *in vitro* cocaine (Chapter 7). In addition, the PKA second messenger pathway was examined as a possible mechanism through which *in vitro* cocaine decreases striatal $GABA_A$ receptor function (Chapter 7).

In conclusion, I hypothesize that striatal GABAergic neurotransmission is reduced in cocaine sensitized rats. This decline in inhibitory neurotransmission would enhance the effect of cocaine stimulated DA neurotransmission and contributes to cocaine sensitization. A decrease in both pre-synaptic striatal GABA release and post-synaptic binding and/or modulation to the striatal $GABA_A$ receptor after cocaine sensitization could diminish control and inhibition of DA release. Either a direct acute or persistent effect of cocaine on $GABA_A$ receptor function could also decrease striatal GABAergic inhibition of DA neurotransmission. Thus, the objective of this dissertation was to evaluate pre-synaptic and post-synaptic striatal GABAergic neurotransmission after cocaine sensitization.

CHAPTER 2
GENERAL METHODS

Animal Care

This dissertation represents a collection of *in vitro* and *in vivo* studies of cocaine on male Sprague-Dawley rats (225-330 g, Harlan). The rats were housed in the University of Florida Animal Care Facility with a 12 h light/dark cycle and allowed rat chow and water *ad libitum.* Rats were cared for according to the guidelines established by the American Association for Accreditation of Laboratory Care and all experimental procedures were approved by the University of Florida's Institutional Animal Care and Use Committee.

Cocaine Sensitization

Treatments were administered to all rats via i.p. injections (7.5, 15 or 20 mg/kg cocaine; or 1.0 ml/kg saline) once daily for 14 consecutive days. These doses of cocaine induce locomotor activation and stereotypy without inducing seizures (Post *et al.*, 1987; Derlet *et al.*, 1992). Behavioral sensitization (see Behavioral Measurements) develops in 75 – 90% of rats and this sensitization lasts for at least one month, but not two (Henry and White, 1995). One day after the last treatment, the rats were killed and their brains were dissected.

Behavioral Measurements

Locomotor and stereotyped behaviors were quantified using a modified 5-point rating scale as described previously (Peris *et al.*, 1990). Each rat was rated by a trained observer, who was blind to the drug treatments. In most cases, I validated the training of the observer with a comparison of test rats co-scored for their cocaine behavioral score. Behavior was measured in test cages, which were located in a separate area from the housing area. The following sterotypy rating scale was based on the Ernst scale (Ernst, 1967): (0) normal quiet behavior, no or little locomotion, no or little sniffing, with or without grooming; (1) exploratory behavior, little locomotion, sniffing over the grid (>4 sec per 10 sec), or infrequent rearing; (2) activated behavior, repeated locomotion, exaggerated sniffing, head bobbing, or rearing; (3) repetitive behavior, very rapid locomotion, repetitive head bobbing, rearing, gnawing or licking; and (4) intense behavior, restricted locomotion, intense head bobbing, or gnawing.

The rats were injected with saline (1.0 ml/kg) once daily for three days prior to actual drug treatments. On the first (day 1), seventh (day 7) and last (day 14) drug treatment days, behaviors were rated. Ten min prior to treatment, rats were placed in their testing cages. The cages were cylinders of 7 mm wire mesh, 30 cm in diameter and 37.5 cm high. Rat behavior was measured at different frequencies and durations in some experiments. In general after treatment, the rats were rated for 10 sec every 2.5 or 5 min for 60 or 90 min. The behavioral scores during the 10 min preceding the treatment were eliminated from the total score because these scores do not reflect behavior resulting from the treatment. The total behavioral score was calculated by summing the individual scores into a cumulative total. For example, if a behavioral rating of 1 was observed 5 times (1x5=5) and 2 was observed 4 times (2x4=8), then the total score would be thirteen. If the rating was for every 5 min for 90 min, then there were a total of 19 evaluation periods with a maximal score of 76. If the rating was for every 2.5 min for 60 min, then there would be a total of 25 evaluation periods with a maximal score of 100.

GABA Release

Slice and Tissue Preparation

The method for collecting endogenous GABA release from striatal slices was modified from a previously described study (Mayfield and Zahniser, 1993). Rats were decapitated and the striata, including a section of globus pallidus, were quickly removed and placed into ice-cold modified Krebs' buffer (118 mM NaCl, 24.5 mM $NaHCO_3$, 11.1 mM D-glucose, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 4.0 μM Na_2EDTA, and 100 μM aminooxyacetic acid saturated with 95% O_2/5% CO_2, pH 7.4). Using a McIlwain tissue chopper (Brinkmann), coronal slices (rostral to caudal, 400 μm) were cut and temporarily placed in 5 ml fresh buffer, then transferred to 15 ml of fresh buffer. Slices were then incubated in a Dubnoff Incu-Shaker for 60 min at 34°C with a change of buffer at the end of the initial 30 min.

Superfusion

Seven glass superfusion chambers in parallel, submerged in a 34°C bath, made up the superfusion system. At the end of incubation, random slices were placed into the superfusion chambers for measurement of endogenous or radiolabeled release (2 slices/chamber, 3-4 chambers/animal). Slices were then superfused with modified Krebs' buffer (constant 95% O_2/5% CO_2 aeration) at a rate of 1 ml/min for 60 min before superfusates were collected. After the recovery period, superfusate was collected in fractions of 5 ml/5 min for 10 min to establish baseline. Next, slices were superfused using a 30 mM K^+ solution (modified Krebs with K^+ replacing Na^+) for 5 min. Superfusates were collected for 25 min and then stored at 4°C until lyophilization. Slices were collected at the end of perfusion and stored at -80°C.

[^{3}H]-GABA Release

The effects of repeated cocaine treatment on basal and stimulated [^{3}H]-GABA release were measured from superfused striatal slices as described previously (Peris and Scott, 1993). Striatal slices were prepared as described above except they were incubated with 35 nM [^{3}H]-GABA (39.6 Ci mmol^{-1}, NEN Life Science Products, Boston, MA) for 30 min after the first initial incubation of 30 min. Slices were superfused with oxygenated Krebs' buffer until stable basal levels were attained (60 min). Superfusates were then collected for a 20 min basal period. Striatal slices were exposed to a 30 mM K$^+$ solution for 5 min. Superfusates were collected for an additional 30 min before slices were solubilized for determination of total tissue tritium. Radioactivity in superfusates and tissue samples was determined by liquid scintillation counting.

GABA Quantification by HPLC-EC

Superfusates from endogenous release experiments were lyophilized using a SpeedVac (Savant Instruments Inc., Farmingdale, NY) and then stored at 4°C. Samples were quantified by isocratic HPLC with electrochemical detection as described previously with slight modifications (Dawson *et al.*, 1989; Wallace and Dawson, 1990). Essentially, samples were resuspended in mobile phase, and derivitized with o-pthalaldehyde/mercaptoethanol reagent for 3 min prior to injection. In some experiments, an internal standard of DL-α-amino-n-butyric acid (DL-ABA) (80 ng/sample) was added to each superfusate fraction prior to lyophilization. Fractions from all slices were measured from the 5 min period during stimulation until endogenous GABA was no longer detectable. In previous measurements, no detectable GABA was measured during the washout period preceding the stimulation.

The HPLC set-up consisted of a PM-11 Solvent Delivery System (Bioanalytical Systems, W. Lafayette, IN; BAS) with a 7125 Rheodyne sample injection valve with a 50 μL sample loop and Ranin Microsorb-MV C18 column (3μm, 4.6mm x 10 cm) in a column heater at 30°C. Electrochemical detection was accomplished by LC-4B detector (BAS) at 0.75 V with sensitivity of 50 nA at 0.1 Hz filtering with TL-5 glassy carbon electrode and recorded on HP-3390A Integrator (Hewlett Packard, Avondale, PA). GABA mobile phase consisted of 0.08 M NaH_2PO_4, 0.269 mM Na_2EDTA, tetrohydrofuran 12.0%, v/v, acetonitrile 12.0%, v/v, and 2.0% propanol-1 at a pH of pH 5.6.

At a flow rate of 1.21 ml/min, the typical retention times for GABA was 6.8 min and DL-ABA was 4.8 min. Figure 2-1 shows typical chromatograms from HPLC-EC for GABA and DL-ABA.

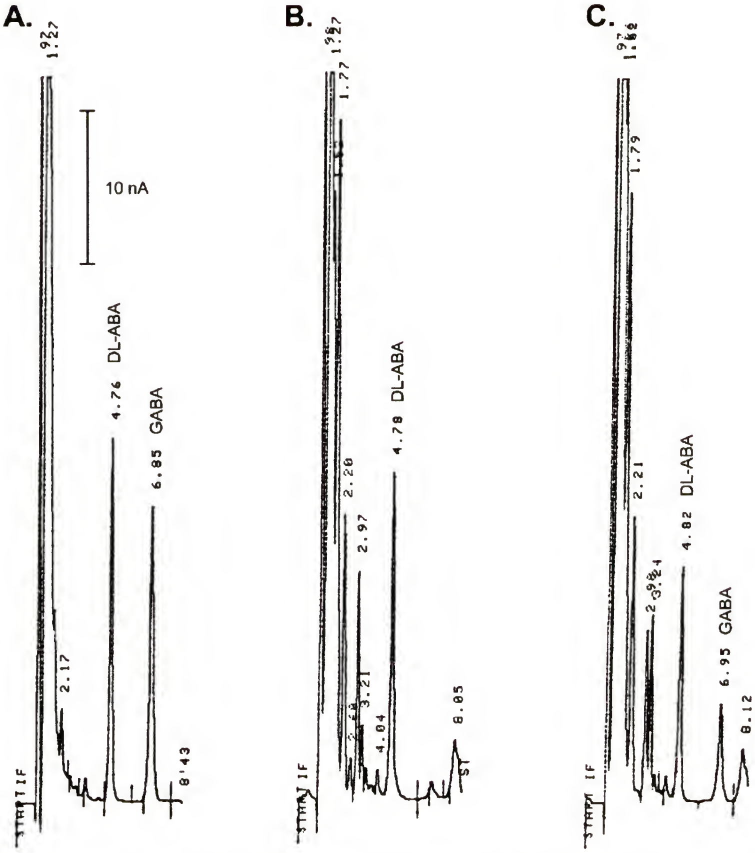

Figure 2-1 Example HPLC-EC Chromatogram. (A) Chromatogram of GABA (5 ng) and DL-ABA (5 ng). (B) Chromatogram of slice superfusate under basal conditions. (C) Chromatogram of a slice superfusate after stimulation; estimated GABA concentration was 2.36 nmol/mg protein. DL-ABA is the internal standard. The minimal detectable concentration was 0.28 nmol/mg protein.

Determination of GABA and Protein Concentration in Striatal Slices

For quantification of GABA and protein, tissue slices were lysed with 1.0 ml of 0.05% Triton-X 100 in distilled water and sonicated with a W-10 Sonicator Cell Disrupter (Heat Systems Ultrasonic, Plainview, NY) for 10 sec. The sample was centrifuged at a 10300 x *g* for 10 min and the supernatant was analyzed for GABA content of the slices as above. Total tissue endogenous GABA content was calculated as the cumulative endogenous GABA released plus the final endogenous GABA remaining in the tissue slice after superfusion. The slice pellet was solubilized in 1 N NaOH and neutralized with 1 N HCl prior to determining the protein content by using a protein-dye binding assay (Bradford, 1976).

GAD Activity

Striatum was homogenized in a 0.5 M HEPES (pH 7.0) buffer with 10 mM 2-amino-ethyl-isothiouronium bromide, 200 μM pyridoxal phosphate, 20 μM gabaculine, and 0.5% Triton X-100 (Buffer A). For the assay of GAD activity, homogenate was added to Buffer A with a final concentration of either 4.5 or 15 μM L-glutamic acid. Homogenate was then allowed to incubate for 30 min at 37°C. Trichloroacetic acid (TCA) was added at the end of the incubation to end the assay. For tissue blanks, TCA was added prior to incubation. For measuring the effect of *in vitro* cocaine, cocaine (1 – 1000 μM) was added with Buffer A before incubation. Samples were then centrifuged two separate times at 4000 x *g* for 20 min. Supernatant was decanted and stored at 4°C until evaluation for GABA by HPLC-EC .

GABA-Dependent $^{36}Cl^-$ Uptake In Brain Microsacs

Microsac Preparation

The methods for microsac preparation and Cl^- uptake have been described previously (Harris and Allan, 1985; Peris, 1993; Peris, 1996). Brains from freshly killed rats were rapidly dissected and homogenized, using a Potter-Elvehjem tissue grinder (Wheaton), in 5.0 ml of ice-cold Ca^{2+} buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1mM $CaCl_2$, and 10 mM HEPES, pH 7.5 with Tris-base, with 10 μM antipain, 10 μg/μl aprotinin and 10 μM leupeptin). Homogenates were centrifuged at 900 x *g* for 15 min using a Sorvall SA600 rotor; supernatants were discarded; and each pellet was resuspended in 5.0 ml buffer. The samples were then centrifuged again at 900 x *g* for 15 min and the final pellets (microsacs) were suspended in assay buffer to yield 800-1600 μg of protein per ml.

Stimulated $^{36}Cl^-$ Uptake

Microsacs in suspensions of 200 μl were pre-incubated for 5 min at 34°C. After pre-incubation, 200 μl of a solution containing $^{36}Cl^-$ (1.08-1.18 μCi μl^{-1}, NEN Life Sciences Products) and treatment drugs were added and vortexed for 3 sec (uptake period). The 3 sec period for stimulated $^{36}Cl^-$ uptake eliminates the effect caused by receptor desensitization (Harris and Allan, 1985). Assays with drug pretreatment included the drug in the 5 min pre-incubation period as well as during the uptake period. Uptake was terminated by addition of 4.8 ml ice-cold Ca^{2+} buffer with 100 μM picrotoxin; followed by vacuum filtration (Hoefer manifold, Hoefer Scientific Instruments, San Francisco, CA) onto a 2.4 cm Whatman GF/C filter. Two additional washes with 5 ml of Ca^{2+} buffer with picrotoxin were applied onto the filter. After the addition of Scintiverse TM (Fisher), the amount of $^{36}Cl^-$ retained on the filter was determined by liquid scintillation. Stimulated uptake was defined as the amount of $^{36}Cl^-$ taken up while agonist was present minus the amount of $^{36}Cl^-$ taken up when agonist was not present. GABA (10 – 100 μM) or muscimol (3 – 30 μM) were used as the $GABA_A$ receptor agonists. Cocaine was added at concentrations ranging from 0.1 – 30 μM. Clonazepam is a benzodiazepine that increases $GABA_A$ receptor function in the presence of GABA or muscimol. Clonazepam (10 or 20 μM) was used in the assays. A water-soluble forskolin derivative (7-deacetyl-7-O-(N-methylpiperazino-γ-butyryl (compound 3 from (Laurenza *et al.*, 1987)), Calbiochem-Novabiochem Corp, San Diego, CA) was used to activate AC. Analogs of cAMP were used to simulate cAMP activity: 8-(4-chlorophenylthio)-cAMP sodium (8-CPT cAMP (0.1 – 1.0 mM), RBI, Natick, MA), 8-bromoadenosine-3', 5'-cAMP (8-Br cAMP (0.5 – 1.5 mM), RBI, Natick, MA), N^6, 2'-O-dibutyryladenosine

3', 5'-cAMP (Bt_2 cAMP (0.5 – 1.5 mM), Sigma, St. Louis, MO). These analogs have been shown to inhibit $GABA_A$ receptor function (Heuschneider and Schwartz, 1989).

Quantitative Receptor Autoradiography

Receptor binding was determined by quantitative autoradiography (QAR). After behavioral sensitization, rats were killed 24 hr after their last injection. Brains were rapidly removed and frozen in powdered dry ice. Frozen brains were stored at -80°C for three to five months until slicing. Brains were sectioned coronally (15 μM) using a cryostat. Two regions of the brain were identified and isolated according to coordinates by Paxinos and Watson (Paxinos and Watson, 1993). The first slice contained the head of the STR, CTX and NAc. The second slice contained the body-tail of the STR and the CTX. Duplicate sections were thaw mounted onto gelatin coated glass slides. Brain sections were stored for up to 5 months in -80°C until radioactive receptor binding assays were performed.

[^{3}H]-Muscimol Binding

Tissue mounted sections were brought to room temperature and allowed to completely dry. The buffer for this experiment was 0.31 M Tris-citrate (pH 7.1). The wash incubation consisted of two 20 min incubations at 4°C. 30 nM [^{3}H]-muscimol (20 Ci mmol^{-1}, NEN Life Science Products) binding was for 90 min at 4°C. Non-specific [^{3}H]-muscimol binding was determined by 200 μM GABA. In a separate experiment, 30 nM [^{3}H]-muscimol was co-incubated with 1 μM flunitrazepam for 90 min at 4°C. Non-specific [^{3}H]-muscimol binding, which also contained 1 μM flunitrazepam, was determined by 200 μM GABA. After total and non-specific binding, slides were washed in Tris-citrate buffer for 1 min at 4°C and then dipped in distilled H_20 for 1 sec. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm (Amersham) for 6 weeks. Film was developed using D-19 (Kodak) for 4 min, stop bath for 1 min and fix bath for 4 min. Finally, the film was washed for 10 min in H_20 and then allowed to dry.

[^{3}H]-Flunitrazepam Binding

Tissue mounted sections were brought to room temperature and allowed to completely dry. The buffer for this experiment was Ca^{2+} buffer (see Microsac preparation). The wash incubation consisted of one 20 min incubation at 4°C. 3 nM [^{3}H]-flunitrazepam (82 Ci $mmol^{-1}$, NEN Life Science Products) binding was for 30 min at 4°C. Non-specific [^{3}H]-flunitrazepam binding was determined by 3 µM clonazepam. In a separate experiment, 3 nM [^{3}H]-flunitrazepam was co-incubated with 200 µM GABA for 30 min at 4°C. Non-specific [^{3}H]-flunitrazepam binding was also determined with both 200 µM GABA and 3 µM clonazepam. After total and non-specific binding, slides were washed in buffer for 6 min at 4°C and then dipped in distilled H_20 for 1 sec. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm (Amersham) for 4 weeks. Film was developed using D-19 (Kodak) for 4 min, stop bath for 1 min and fix bath for 4 min. Finally, the film was washed for 10 min in H_20 and then allowed to dry.

Film Analysis

The brain images were visualized using a light box (Northern Light, Imaging Research Inc., Canada), and digitized using a video camera (Sierra Scientific 4030 solid state video camera) with a Nikon 55 mm Micro lens (Nikon, USA). The image was then captured onto a personal computer (Compaq Deskpro 386/20, Compaq, USA) and analyzed with quantitative imaging software (BRS2, Imaging Research Inc., Canada). Film density was normalized using [^{3}H]-Microscales (Amersham), which has been calibrated to a known concentration of protein. Specific binding was determined by subtracting non-specific binding from total binding.

Data Analysis

Results are expressed as mean values ± SEM; N indicates the number of animals used. The sums of the rankings for each rat from behavioral experiments on the first and last days were analyzed by the Wilcoxon signed-rank test. Mann Whitney U was used to determine within group differences. Unpaired *t*-tests were used to analyze cumulative endogenous GABA release, [^{3}H]-GABA release and GAD activity. Multiple *t*-test comparisons were adjusted with the Bonferroni correction. ANOVA was used to analyze [^{3}H]-muscimol binding, enhanced [^{3}H]-muscimol binding, [^{3}H]-flunitrazepam binding, enhanced [^{3}H]-flunitrazepam binding, GABA stimulated ^{36}Cl$^-$ uptake, and muscimol stimulated ^{36}Cl$^-$ uptake. When appropriate, the Bonferroni-Dunn analysis was used as the *post-hoc* follow up test.

CHAPTER 3
ENDOGENOUS GABA RELEASE IS REDUCED IN THE STRIATUM OF COCAINE-SENSITIZED RATS

Introduction

Repeated cocaine exposure results in a behavioral phenomenon known as cocaine sensitization, which consists of increased behavioral stereotypy (head bobbing, repetitive motions) and locomotion. The duration of cocaine sensitization after withdrawal is persistent, lasting days (Liu-Chu *et al.*, 1985) to weeks (Post *et al.*, 1987; Henry and White, 1995). The mechanism by which cocaine causes such persistent behavioral sensitization is not completely understood. Although the immediate actions of cocaine are to inhibit DA re-uptake by the DAT and to increase DA concentration extracellularly, changes in striatal dopaminergic transmission alone cannot account for the behavioral changes seen after repeated cocaine (see Chapter 1 for details). Striatal release of endogenous DA by cocaine is elevated in a dose dependent manner seven days after cocaine withdrawal (King *et al.*, 1993), but not for shorter time points (Peris *et al.*, 1990). In summary, except for a change in stimulated DA release, there are no clearly persistent changes in striatal dopaminergic transmission that could account for enhanced stereotypy after repeated cocaine at all time points at which the behavior occurs. This equivocal relationship between dopaminergic pathways and behavioral sensitization suggest that other neurotransmitter pathways may be involved in mediating behavioral changes associated with repeated cocaine exposure.

Dopaminergic and GABAergic neurons in the striatum form circuits that balance and oppose the other's actions (see Chapter 1 for details). Striatal DA release results in a net excitatory effect on locomotion via the striatonigral pathway and a net inhibitory effect on movement via the striatopallidal pathway (Sommer *et al.*, 1996). GABA neurotransmission regulates and inhibits striatal dopaminergic transmission (Reid *et al.*, 1990a; Reid *et al.*, 1990b; Gruen *et al.*, 1992; Smolders *et al.*, 1995; Morgan and Dewey, 1998). Therefore, a decrease in the tonic inhibitory role of striatal GABA could contribute to enhanced striatal dopaminergic output caused by repeated cocaine.

Repeated cocaine treatment resulting in behavioral sensitization has been shown to reduce both the number and function of $GABA_A$ receptors in rat striatum (Pecins-Thompson and Peris, 1993; Peris, 1996). While post-synaptic striatal GABA transmission is reduced by cocaine treatment, studies conducted to date do not determine if cocaine-induced decreases in GABA transmission also include pre-synaptic striatal changes. If a decrease is found in pre-synaptic striatal release of GABA, this could indicate an overall decrease in GABA transmission and its subsequent inhibition of the DA signal. Alternatively, a compensatory response to decreased $GABA_A$ receptors might include increased GABA release, thereby precluding an overall decrease in GABAergic transmission in cocaine treated rats. I hypothesize that repeated cocaine exposure results in a decrease in the inhibitory GABA signal in the striatum, thereby contributing to enhanced striatal output.

In this study, both endogenous and radiolabeled GABA release were examined in superfused striatal slices of sensitized and non-sensitized rats. The release of endogenous and radiolabeled GABA has been previously shown to measure different intraterminal pools (Szerb, 1983). Since the mechanisms regulating these two distinct types of GABA release may be discretely affected by repeated cocaine, it would be beneficial to document the differences between endogenous and exogenous GABA release in cocaine-sensitized rats. The effects of repeated cocaine on the development of sensitization is known to be dose-dependent (Post and Rose, 1976; Shuster *et al.*, 1977). The effects of cocaine dose on striatal endogenous GABA release is not known, but will be examined here. Previously, it has been shown that there is a reduction in number and function of the $GABA_A$ receptor in the striatum under similar cocaine-treatment conditions (Pecins-Thompson and Peris, 1993; Peris, 1996). Expanding on our previous findings, the results obtained show that striatum from cocaine-sensitized rats also releases less endogenous GABA, indicating an overall decrease in GABA inhibitory transmission. The decrease in striatal endogenous GABA release also appears to be cocaine dose-dependent.

Methods

Animals and Treatment

Male Sprague-Dawley rats were used and housed as described in Chapter 2. After three daily saline injections (1.0 ml kg^{-1} i.p.) in the home cages, the drug treatment regimen started. In two sets of experiments, animals received either 1.0 ml kg^{-1} saline i.p. (Group SI or SII, N = 6 and 9), or cocaine at one of two doses once daily for 14 days. In the first experiment, rats received 7.5 mg kg^{-1} cocaine i.p. (Group C7, N = 6), while in the second, they received 15 mg kg^{-1} cocaine i.p. (Group C15, N = 9). The rats for Group C15 were prepared at two separate times. Rats were killed 24 h after their last injection to obtain striatal tissue. Endogenous GABA release was also measured in rats given either a single dose of saline (0.1 ml kg^{-1}, i.p., N = 4) or cocaine (15 mg kg^{-1}, i.p., N = 4). [^{3}H]-GABA release was measured in a separate experiment. In this experiment, the rats received the same treatment for 14 days as above (Group S, N = 6; and Group C15, N = 6).

Behavioral Rating

Behavior was rated as described in Chapter 2 on Day 1 and 14. Behavior was rated for 10 sec every 5 min for 90 min. Rats were determined to be sensitized if there was a significant increase in cumulative behavioral scores when Day 14 was compared to Day 1.

GABA Release from Superfused Striatal Slices

The levels of GABA, $[^3H]$-GABA and protein from the striatum of treated rats were determined as described in Chapter 2. Briefly, striatum of treated rats were removed, slices prepared and perfused in the superfusion system to either measure endogenous or exogenous GABA release. Slices were perfused with a 30 mM K^+ solution for 5 min to stimulate release of GABA. Endogenous GABA release was measured by HPLC-EC. Exogenous GABA was added using $[^3H]$-GABA and measured by liquid scintillation counting. Endogenous GABA from striatal slices was measured also by HPLC-EC after extraction from tissue by sonication and centrifugation. Proteins were measured by Bradford assay.

Data Analysis

Results are expressed as mean values ± SEM; N indicates the number of animals used. The sums of the rankings for each rat from behavioral experiments on the first and last days were analyzed by the Wilcoxon signed-rank test. Mann Whitney U was used to compare within group differences. Unpaired *t*-tests were used to analyze cumulative endogenous GABA release, and $[^3H]$-GABA release. Multiple *t*-test comparisons were adjusted with the Bonferroni correction.

Results

Behavioral Sensitization to Cocaine

Locomotor and stereotypic behaviors were rated after injections of saline or cocaine on Days 1 and 14. On Days 1 and 14 (Figure 3-1A), behavioral ratings of Groups S and C7 were not significantly different across days. When behavioral ratings of Groups S and C15 were examined (Figure 3-1B), Group C15 showed a significant difference from Day 1 to Day 14 ($p < 0.01$). Striatal tissue from these rats was assayed for endogenous GABA release. In this study, two separate groups of Group C15 rats were prepared. In the first Group C15 rats, all of the cocaine treated rats showed higher behavioral scores when Day 14 is compared to Day 1 (data not shown). All rats (6) in the second Group C15 showed an increase in behavioral score when Day 14 is compared to Day 1 (data not shown). The behavioral scores between Group C15 rats on Day 1 and Day 14 were not significantly different and were combined for ease of comparison. Due to experimental error, release data from 3 pairs of rats in the first Group C15 was not included, thus the final Group C15 had a number of 9 rats.

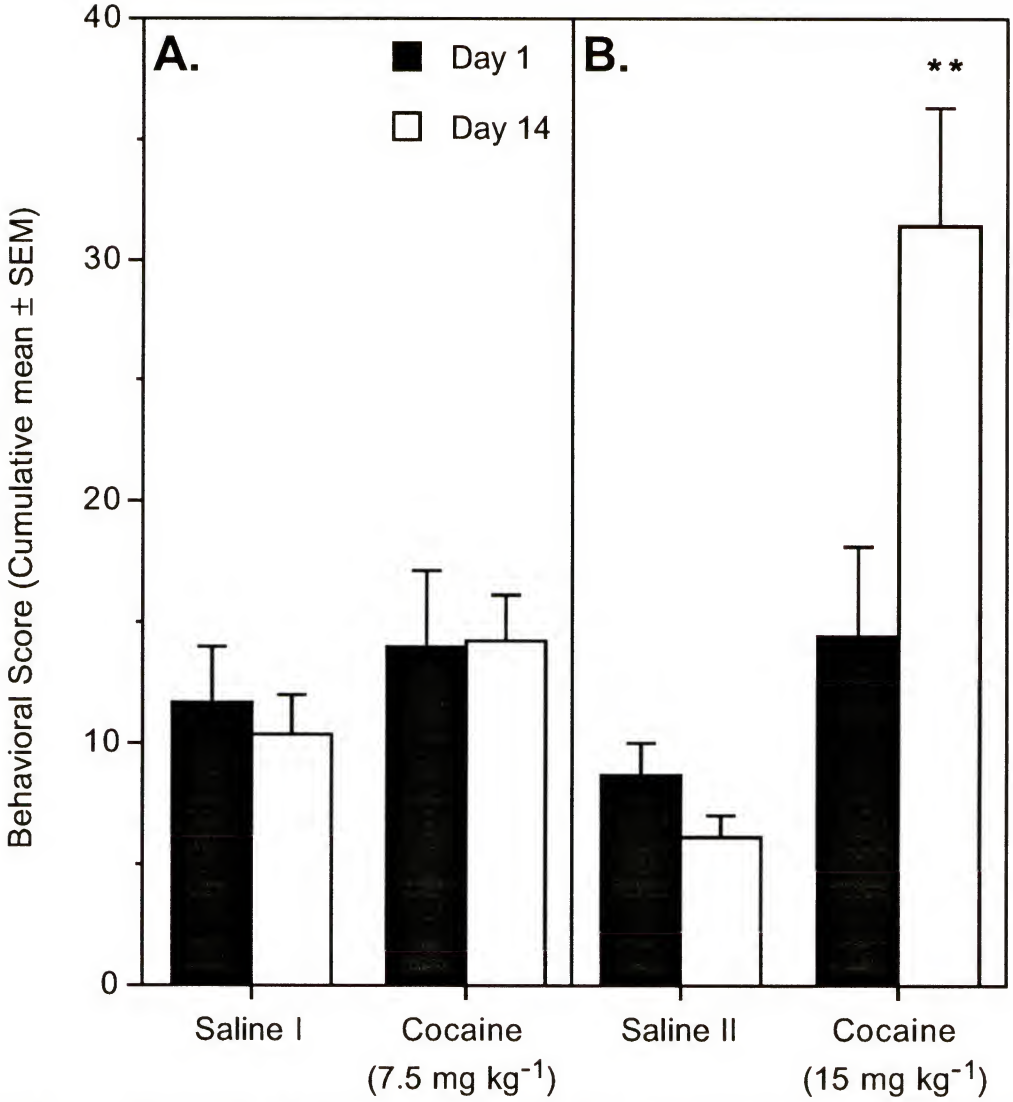

Figure 3-1 Cumulative behavioral scores of saline or cocaine treated rats on Day 1 and 14. A) Rats were treated once daily for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (7.5 mg kg^{-1}). B) Rats were treated once daily for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}). Behavior was rated on Day 1 and 14 for 10 sec every 5 min for 90 min. Daily cocaine treatment with cocaine (15 mg kg^{-1}) led to significantly greater behavior on Day 14 compared to Day 1 ($p < 0.01$). The effect of cocaine on behavioral sensitization appears dose-dependent because cocaine (7.5 mg kg^{-1}) did not result in sensitization, while cocaine (15 mg kg^{-1}) resulted in sensitized behavior. The cumulative behavioral scores within treatment groups were compared between Day 1 and 14 using the Wilcoxon signed-rank test. Data are expressed as the cumulative mean ± SEM for Group SI (N = 6), Group SII (N = 9), Group C7 (N = 6), and Group C15 (N = 9). $^{**}p < 0.01$ compared to group Day 1.

Release of Endogenous GABA

Striatal slices from rats in Groups S, C7 and C15 were assayed for their ability to release endogenous GABA during superfusion. The two saline treated groups displayed no significant differences between each other and were therefore collapsed into a single group for ease of viewing only. Figure 3-2 shows the time course of endogenous GABA release from stimulated striatal slices of the treated rats. For all groups, there was a peak of endogenous release of GABA in the 10 min collection. Although there was a trend for decreased endogenous release in Group C15 at each time point, there were no significant differences between groups. Endogenous GABA was not detectable in superfusates earlier than 5 min and later than 15 min. When cumulative GABA release was calculated for each treatment (Figure 3-3), Group C15 was significantly lower compared to Group S ($p < 0.018$). There was no change in GABA release when Group S was compared to Group C7. Total endogenous GABA content in the slices was not significantly different among the three treatment groups (Figure 3-4). Groups S and C7 were similar and Group S was slightly, but not significantly higher ($p > 0.15$) than Group C15. There was no difference in percentage of total endogenous GABA release (GABA release/total endogenous GABA) between Group S (3.99 ± 0.99 %), Group C7 (3.97 ± 2.18%) and Group C15 (3.71 ± 2.21%).

When rats were given only one saline or cocaine injection, there was no difference in endogenous GABA release between saline (12.23 ± 1.45 nmol mg protein^{-1}) and cocaine (13.14 ± 1.35 nmol mg protein^{-1}) treated rats. Total endogenous GABA content in slices of acutely treated rats was not different between saline (171.20 ± 7.71 nmol mg protein^{-1}) and cocaine (185.67 ± 16.81 nmol mg protein^{-1}) treated rats.

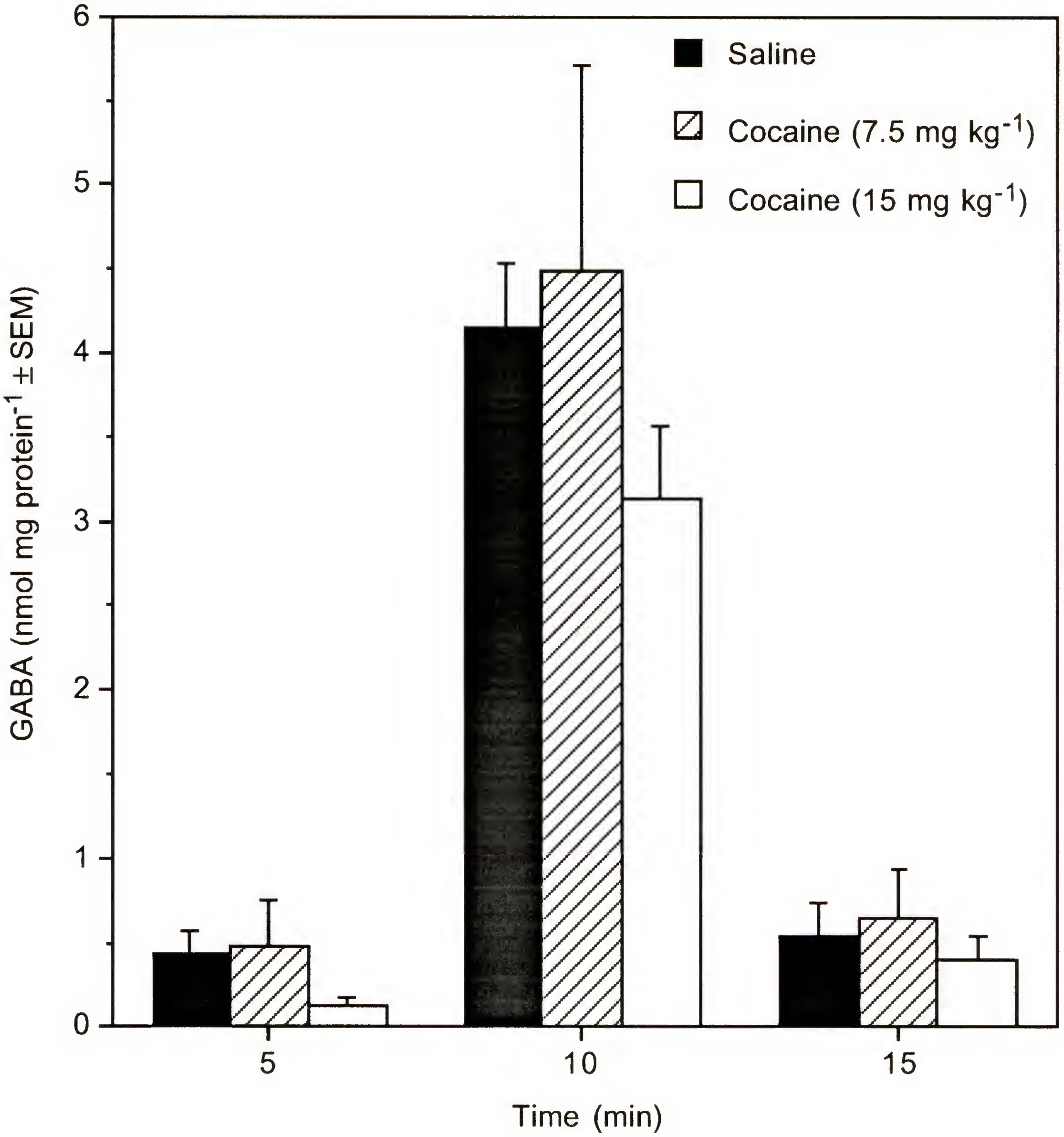

Figure 3-2 Time course of endogenous GABA release from superfused striatal slices. Rats were treated with either saline or cocaine (7.5 or 15 mg kg^{-1}) once daily for 14 days. Stimulated endogenous GABA release from striatal slices of treated animals was evoked by superfusion for 5 min with 30 mM K^+ starting at time 0. Endogenous GABA was not detectable in the first fraction (0 min) nor in superfusates after 20 min. Endogenous GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM for N = 6 – 9. Release was measured in at least duplicate slices from each rat. ANOVA was used to compare groups, but there was no significant difference between groups at any time period.

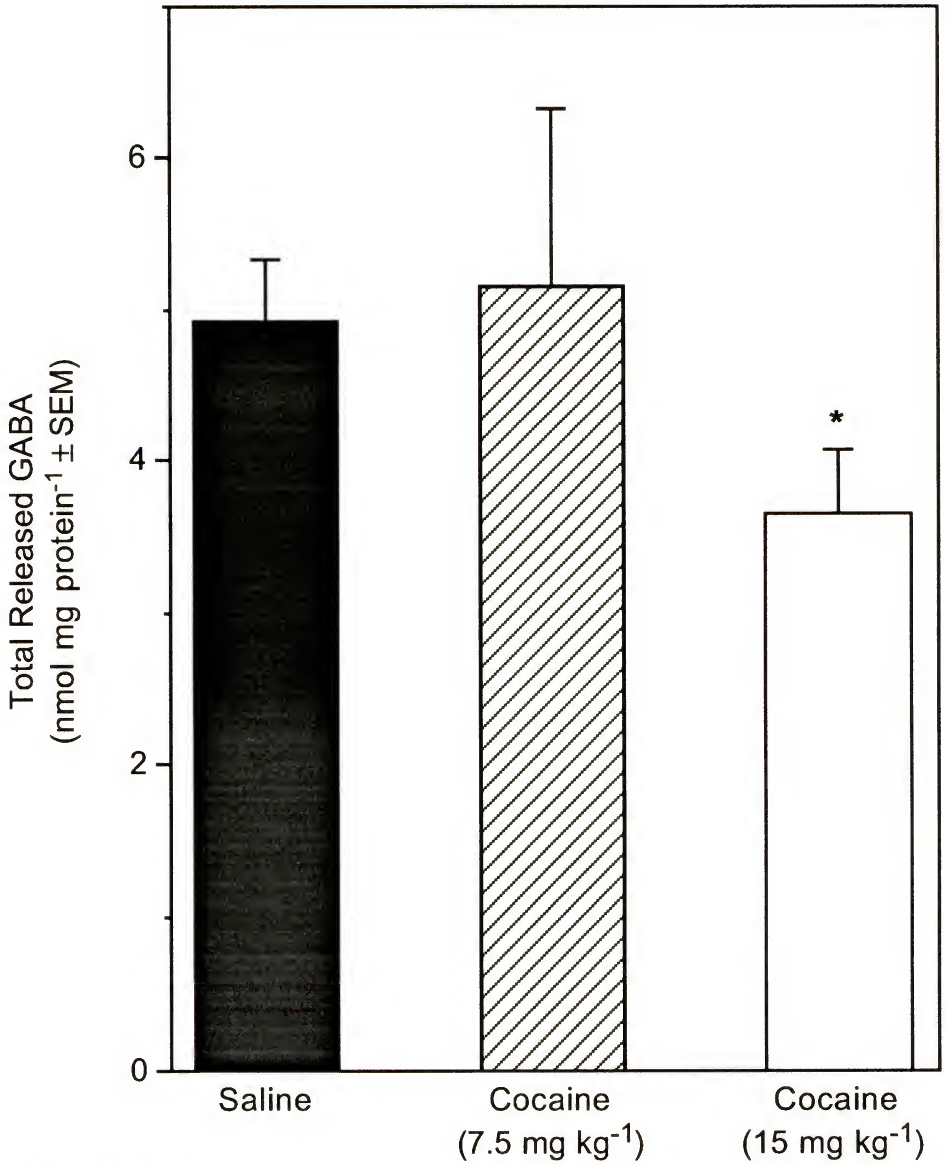

Figure 3-3 The effects of repeated cocaine on cumulative release of endogenous GABA from rat striatal slices after K^+ stimulation. Striata from saline and cocaine treated rats from Figure 3-1 were used. Stimulated endogenous GABA release from striatal slices was evoked by superfusion for 5 min with 30 mM K^+. Cumulative stimulated release (average sums of 3-4 fractions for each slice) was calculated for each treatment. GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM for Group SI (N = 6), Group SII (N = 9), Group C7 (N = 6), and Group C15 (N = 9). $^*p < 0.025$ compared to Group SII. Unpaired *t* test was used for comparison. For graphical purposes, Group S represents all saline treated animals.

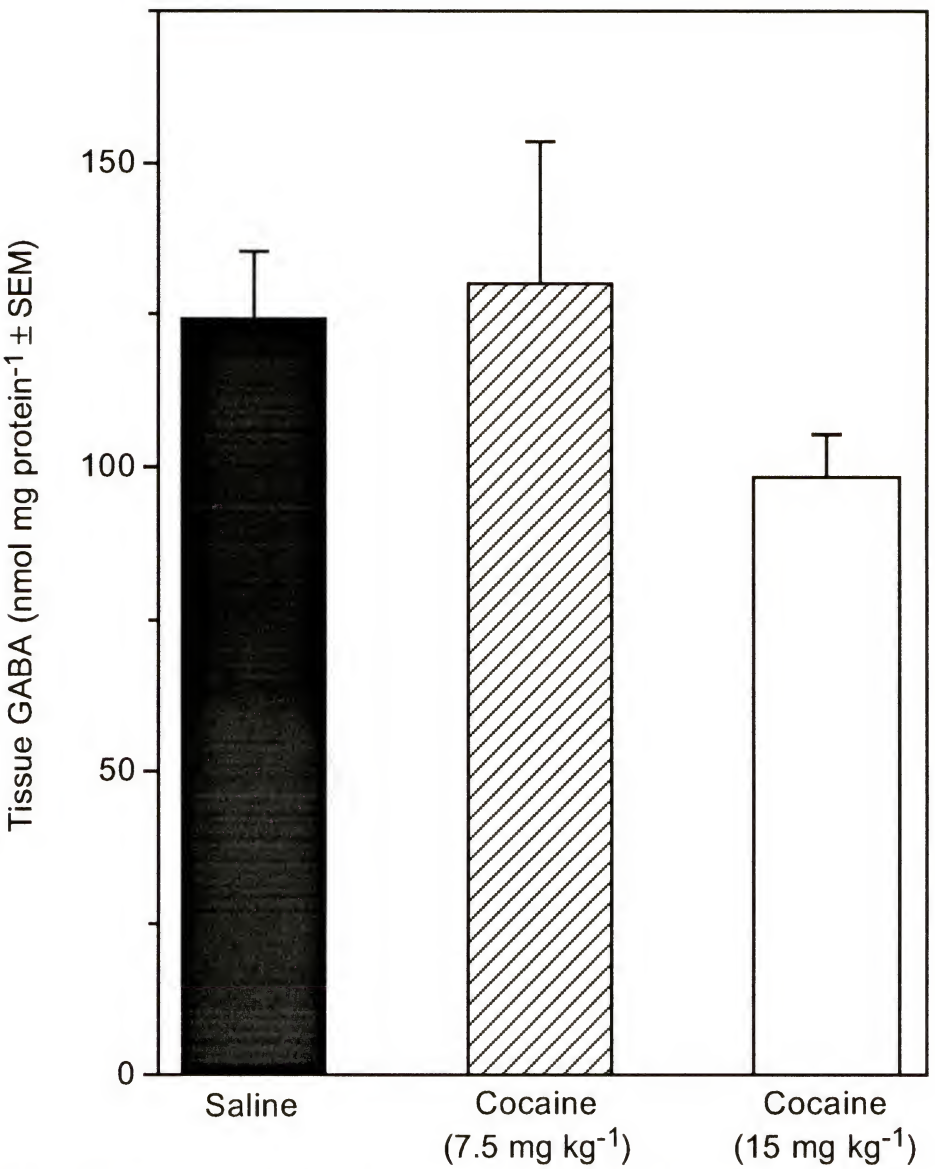

Figure 3-4 The effects of repeated cocaine on total GABA content of rat striatal slices. Total GABA was calculated as the amount of endogenous GABA released from each slice plus the GABA content remaining in the slice after release. GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM for Group SI (N = 6), Group SII (N = 9), Group C7 (N = 6), and Group C15 (N = 9).

Release of [^{3}H]-GABA

Next, we measured [^{3}H]-GABA release from pre-loaded striatal slices of similarly treated rats 24 hr after their last injection. There was no difference in basal [^{3}H]-GABA release between Group S (0.40 ± 0.02 % total tritium) and Group C15 (0.39 ± 0.02 % total tritium). Potassium stimulated [^{3}H]-GABA release from Group C15 (20.86 ± 0.57

% total tritium) was slightly, but significantly, higher ($p < 0.01$) than stimulated [^{3}H]-GABA release from Group S (18.11 ± 0.79 % total tritium). There was no significant difference in the total tissue [^{3}H]-GABA uptake between saline (326505 ± 57123 DPM) and cocaine (355784 ± 63769 DPM) treated rats.

Discussion

Previously, it has been shown that cocaine sensitization results in decreased number and function of $GABA_A$ receptors in the striatum (Pecins-Thompson and Peris, 1993; Peris, 1996). Generally, conditions that result in a decrease in the number and/or function of receptors usually involve prolonged exposure of receptors to their endogenous neurotransmitter. Thus, the previously reported findings may have been thought to be the result of an increase in GABA release in striatum. On the contrary, our present findings indicate that repeated cocaine exposure, sufficient to cause behavioral sensitization, induces a decrease in endogenous striatal GABA release. Thus, behavioral sensitization paradoxically causes a decrease in both endogenous GABA release and $GABA_A$ receptors in striatum, neither of which are compensated for in the customary way. Intraterminally, the modest decrease of 30% in release could have a profound decrease in GABA inhibition especially in combination with reduction of 30% in $GABA_A$ receptor function and number as we have found previously.

When rats were given a lower dose of cocaine for two weeks or a single treatment of 15 mg kg^{-1} cocaine, there was no evidence of increased stereotypic behavior. These data are consistent with previous findings that cocaine sensitization is dependent on both dosage of cocaine and number of exposures (Post and Rose, 1976; Shuster *et al.*, 1977). Rats receiving cocaine at either the lower dose or a single exposure of the higher dose also do not exhibit decreased striatal GABA release indicating that this neurochemical change may be related to the presence of behavioral sensitization. However, it should be noted that the behavior scale measures both locomotor activity and stereotypy and may be insensitive in measuring sensitization with 7.5 mg kg^{-1} cocaine. It will be important to determine whether other techniques could detect sensitization with 7.5 mg kg^{-1} cocaine treatment. Over the 90 min duration of behavioral rating, no Group C15 rat reached the maximal rating on the behavioral scale. The distribution of ratings were on the lower end of the scale (2), with few scores at 3. Thus, Group C15 rats did not show maximal stereotypic behavior. It is possible that if the animals had shown more stereotypic behavior that there may have been greater changes in both endogenous and exogenous GABA release.

The decrease in endogenous GABA release, together with those indicating a decrease in striatal $GABA_A$ receptor number and function after repeated cocaine (Pecins-Thompson and Peris, 1993; Peris, 1996), indicate that GABAergic transmission in striatum is decreased both pre- and post-synaptically in cocaine-sensitized rats. A decrease in endogenous GABA release after cocaine may depend on a particular brain region and its function. For example, in the dorsolateral septal nucleus (DLSN), repeated cocaine treatment leads to reduced $GABA_B$ receptor function and increased pre-synaptic GABA release (Shoji *et al.*, 1997). These results are not surprising because the limbic system, which includes both the DLSN and the mesocorticolimbic DA pathway, are both strongly implicated in mediating the reinforcing effects of cocaine (Koob, 1992). Tolerance develops to the reinforcing or euphoric effects of cocaine (Emmett-Oglesby *et al.*, 1993), whereas cocaine sensitization develops to the stereotypic effects, which is mediated by the striatum. The diametric effects of repeated cocaine in these brain regions on GABA transmission may help explain why tolerance and sensitization occur.

Although the amount of total tissue endogenous GABA was not significantly different ($p > 0.15$) between Group SII and Group C15, there was a trend towards a reduction (Figure 3-4). Since the percentage of total endogenous GABA released was not different among the treatment groups, this suggests that the trend towards a decrease in tissue GABA may contribute to the decrease in endogenous GABA release. It is unknown if this trend towards a decline in tissue GABA is the result of an acute or chronic reduction of GABA production caused by cocaine. It is also possible that GABA synthesis was decreased during the incubation period preceding GABA release in the striatal slices after repeated cocaine. GABA synthesis after repeated cocaine will be examined in Chapter 4.

In contrast to the 30% decrease in endogenous GABA release, striatal [^{3}H]-GABA release was slightly, but significantly increased (15%) in cocaine-sensitized rats. Thus, endogenously and exogenously derived GABA release appear to be differently affected by cocaine sensitization. This is not surprising, however, because of possible differences in endogenous and exogenous GABA incorporation into intraterminal pools and subsequent differences in release in response to various stimuli (Ryan and Toskoski, 1975; Sellström and Hamberger, 1977; Szerb *et al.*, 1981; Abe and Matsuda, 1983; Szerb, 1983; Lidén *et al.*, 1987). Neuronal GABA exists in several intraterminal pools (see Figure 3-5A): newly synthesized pools (S) and metabolic pools (M) filled via re-uptake pumps. In these experiments, the released endogenous GABA could originate from both the newly synthesized pools and the metabolic pools (Ryan and Toskoski, 1975; Szerb *et al.*, 1981; Abe and Matsuda, 1983; Szerb, 1983). Depletion of either of these pools could contribute to the decrease in endogenous GABA release seen in cocaine-sensitized rats (Figure 3-5B). On the other hand, the source of radiolabeled or exogenous GABA release is solely the metabolic pools filled through re-uptake (Szerb *et al.*, 1981; Abe and Matsuda, 1983; Szerb, 1983). If cocaine sensitization depletes this pool, then incubation with [^{3}H]-GABA might be expected to result in greater filling of these pools compared to naive rats (Figure 3-5B). Therefore, endogenous GABA release would be decreased in the cocaine treated rats; and exogenous GABA release would be increased. Thus, the change in endogenous GABA release may be a better representation of what occurs in striatal neurons compared to [^{3}H]-GABA release.

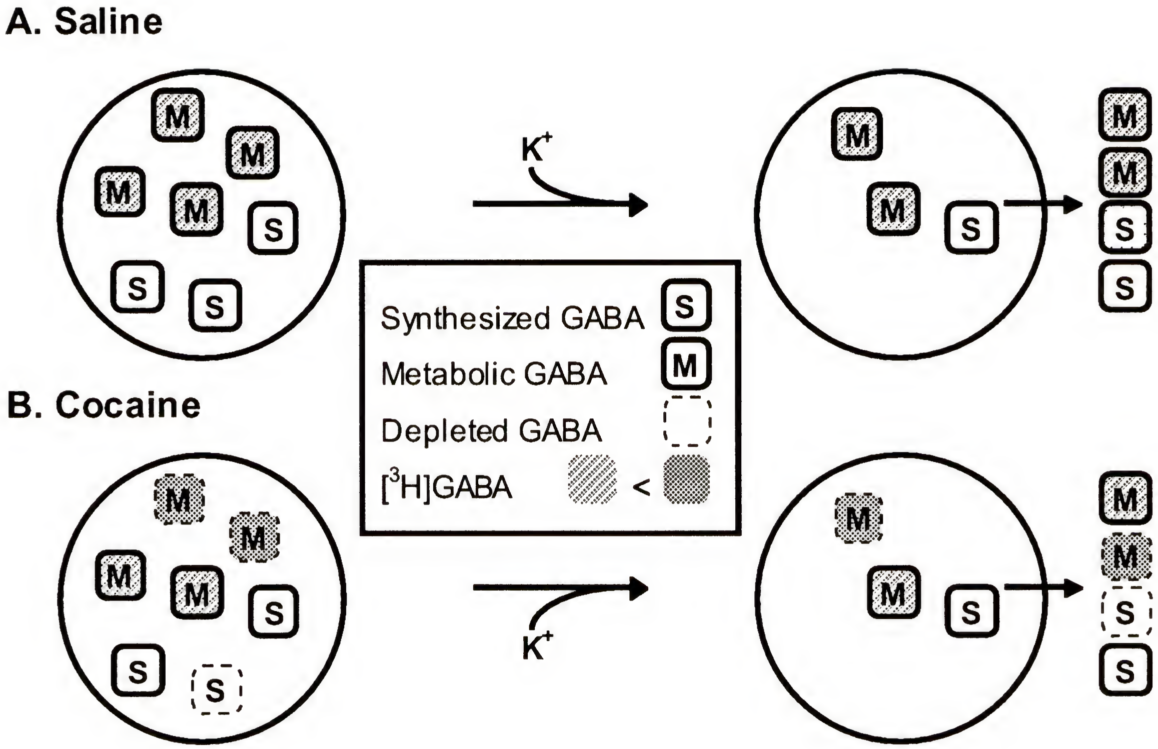

Figure 3-5 Representation of exogenous and endogenous striatal GABA release from saline and cocaine treated rats. A. Saline treated neurons release equivalently filled exogenous and endogenous GABA pools. B. Cocaine treated neurons release depleted and re-filled exogenous and endogenous GABA pools. The amount of detectable [^{3}H]-GABA will be higher because of the additional [^{3}H]-GABA needed to fill the depleted pool. Endogenous GABA will detect both decreases in synthesized and metabolic pools.

An alternative interpretation is that the small magnitude of increase in exogenous GABA may indicate a minor effect. We have observed that exogenous GABA release is not changed from striatal slices 48 hr after withdrawal in cocaine-sensitized rats under different stimulation parameters (data not shown). The effect of cocaine sensitization is also know to be persistent (Liu-Chu *et al.*, 1985; Post *et al.*, 1987; Henry and White, 1995). It will be important to test if the duration of decreased endogenous GABA release is persistent after cocaine withdrawal. In addition, it would be interesting to determine if higher cocaine doses could cause further decreases in endogenous GABA release. Finally, the point where endogenous GABA release first declines would determine the importance of this change to the development of cocaine sensitization. Thus, the persistence, magnitude and initiation of the changes in both types of GABA release must be determined since cocaine sensitization is known to be long lasting.

An additional source of endogenous GABA in these experiments is from glial GABA pools filled via re-uptake pumps. Breakdown of GABA by glial GABA transaminase (GABA-T) is inhibited by aminooxyacetic acid (AOAA), which was present during all release assays. Therefore, GABA was allowed to form a releasable pool in glia under these conditions. GABA release from glia cells is known to occur after K^+ stimulation, however the relative importance is controversial (Sellström and Hamberger, 1977; Neal and Bowery, 1979). At stimulatory concentrations of extracellular K^+ (60 mM), glial release of [^{3}H]-GABA is relatively small and not significant when compared to neuronal [^{3}H]-GABA release (Neal and Bowery, 1979). Even in the presence of Ca^{2+}, the amount of GABA released by the glia cells does not increase to a similar magnitude as when K^+ stimulates neuronal GABA release (Neal and Bowery, 1979). However, it would be important to test under our conditions whether GABA release from glial pools is affected by cocaine sensitization by removing Ca^{2+} from the superfusate buffer.

The effect of repeated cocaine on striatal neurons could also be neurotoxic. It is possible that the integrity of the cell membrane could be affected by repeated cocaine and make the neuron more susceptible to death. If cocaine sensitized striatal slices had greater neuronal death during the period preceding GABA release, then this could account for the decrease in endogenous GABA release. Since exogenous GABA release was not affected in the same way by repeated cocaine, this could exclude some of the problems associated with neurotoxicity. However, the increased exogenous release could be related to neuronal death because of increased “leakiness.” The effects of repeated cocaine has been examined for neurotoxicity in the hippocampus (Goodman and Sloviter, 1993). These authors found that cocaine (40 mg kg^{-1}, i.p.) for five days and three months did not result in hippocampal neuronal cell death (Goodman and Sloviter, 1993). Although the striatum was not examined, it is tempting to speculate that striatal neurons could survive that treatment and also remain viable in this study. However, the viability of the neurons after repeated cocaine before GABA release should be considered in future experiments.

It is not expected that the changes in GABA release after cocaine sensitization are caused by changes in pre-synaptic receptor regulation. $GABA_B$ autoreceptors regulate GABA release in the striatum and DLSN (Mayfield and Zahniser, 1993; Shoji *et al.*, 1997). Cocaine treatment reduces $GABA_B$ autoreceptor function and leads to increased GABA release in the DLSN (Shoji *et al.*, 1997). If cocaine increases the number or function of striatal $GABA_B$ receptors, this could result in decreased striatal GABA release. Additionally, D_2 DA receptor activation inhibits GABA release *in vitro* (Starr, 1987; Bernath and Zigmond, 1989; Retaux *et al.*, 1991) and decreases extracellular GABA concentrations *in vivo* (Reid *et al.*, 1990b; Drew and Ungerstedt, 1991). Repeated cocaine exposure increases the inhibitory influence of D_2 DA receptors on DA release in the striatum (Dwoskin *et al.*, 1988). If D_2 DA receptors on GABA terminals are similarly affected by intermittent cocaine, they could also decrease GABA release. However, it is unlikely that a change in either autoreceptor or heteroreceptor regulation of GABA release could account for the opposite changes in exogenous and endogenous GABA release in cocaine sensitized rats.

A similar argument can be used to eliminate other intraterminal changes that might alter GABA release in cocaine-sensitized rats. If Ca^{2+} dynamics were decreased by intermittent cocaine, this may reduce exocytosis of synthesized GABA. However, these changes would be expected to affect release of both exogenous and endogenous pools of GABA similarly. As stated earlier neuronal GABA release is Ca^{2+}-dependent, the difference between endogenous and [^{3}H]-GABA release may be due to the contributions of both Ca^{2+}-dependent and independent [^{3}H]-GABA release. Determining the

Ca^{2+}-dependent and independent components of [^{3}H]-GABA release should be considered in the future. Increased GABA breakdown by GABA-T is unlikely in our experiments because GABA-T activity was inhibited by AOAA. Additionally, GABA-T activity is not significantly changed by a high concentration of cocaine (Ikeda *et al.*, 1983). The breakdown metabolites of GABA may have also contributed the increase in [^{3}H]-GABA release. However, it has been shown in cortical slices that 95% of [^{3}H]-GABA released was unmetabolized (Peris *et al.*, 1987). AOAA also increases the excitability of neurons and makes them more susceptible for stimulated release (Orrego and Miranda, 1976). The degree to which AOAA contributed to the changes in GABA release of sensitized rats is unknown, but it would be expected to equally affect release regardless of the technique used to measure release.

In summary, I show that there is a decrease in the endogenous GABA release in the striatum of cocaine-sensitized rats. This change in endogenous GABA release may be a better representation of what occurs in striatal neurons compared to [^{3}H]-GABA release. $GABA_A$ receptor function and number has been previously shown to be decreased after repeated cocaine (Peris, 1996 and Pecins-Thompson and Peris, 1993). Thus, repeated cocaine exposure decreases both the pre- and post-synaptic transmission of the inhibitory GABA signal in the striatum. This loss of inhibitory influences may contribute to the enhanced DA neurotransmission in striatum associated with cocaine sensitization.

CHAPTER 4
GLUTAMIC ACID DECARBOXYLASE ACTIVITY AFTER COCAINE SENSITIZATION

Introduction

When an individual first observes the tricarboxylic acid cycle or Krebs cycle, one of the detours that gets little initial attention is the branch off to the amino acid synthesis pathway. It is usually enough to find the key regulatory sites of that distasteful, eternally spinning circuit. However from this lesser attraction of the Krebs cycle, the majority of GABA is produced in the neurons. The path to GABA synthesis begins when α-ketoglutarate from the Krebs cycle is shunted and transaminated to form glutamate. Glutamate serves as the substrate for the enzyme, glutamic acid decarboxylase (L-glutamate-1-decarboxylase; EC 4.1.1.15, GAD). GAD then decarboxylates a carbon chain from the glutamate molecule releasing GABA and CO_2.

To function efficiently, GAD requires both glutamate as a substrate and pyridoxal 5'-phosphate as a cofactor. Pyridoxal 5'-phosphate is one of the active compounds of essential vitamin B_6. Unless there is a dietary deficiency, there is enough pyridoxal 5'-phosphate generated to supply GAD and other dependent enzymes (Martin and Rimvall, 1993). In addition to the supply of glutamate off the Krebs cycle, another pathway supplies the neuron with glutamate. Through the GABA shunt, the breakdown of GABA allows for the indirect recycling of GABA back to glutamate, which is the substrate for GAD (Cooper *et al.*, 1996). The GABA shunt involves the energy created in the break down of GABA by GABA-T for the generation of glutamate from α-ketogluterate. There is some evidence to show that synaptosomes *in vitro* have glutamate concentrations that may be below the K_m for GAD, but whether this occurs physiologically and can regulate GABA production is unknown (Martin and Rimvall, 1993). Thus in a typical neuron, the concentrations of both glutamate and pyridoxal 5'-phosphate are sufficiently higher than the K_m of GAD and fully saturate the enzyme.

GAD is the key enzyme in the formation of GABA. In the brain, the total capacity of GABA synthesis is much higher than the actual rate of GABA synthesis (Martin and Rimvall, 1993). There are two isoenzymes of GAD: GAD_{67} and GAD_{65}. The GAD isoenzymes are quite homologous (in rat 65% identical and 80% similar) (Martin and Rimvall, 1993) and are equally capable of synthesizing GABA. The GAD isoenzymes come from two independent genes located on different chromosomes (Erlander *et al.*, 1991). Since substrate and cofactor are in sufficient supply, regulation of GAD itself becomes the rate limiting step for GABA production. GAD isoenzyme regulation of GABA production is less clear. GABA is located in two recognizable pools: the synthetic and the metabolic (Martin and Rimvall, 1993). GAD_{65} produces the synthetic pool of GABA, which supplies the GABA releasable pool. However, GAD_{67} can also contribute to the synthetic pools of GABA. GAD_{67} is co-localized with and creates the metabolic pool of GABA. The physiologic purpose of the metabolic pool is unclear, but it is speculated to serve as a metabolic intermediate relating to the Krebs cycle (Martin and Rimvall, 1993). Although GAD_{65} and GAD_{67} have been identified, the regulation of the enzymes' transcription, translation and activity is still being characterized.

In addition to the possible long term regulation of the two isoforms, there is a more direct regulation of the enzyme by the presence or absence of bound active pyridoxal 5'-phosphate (Martin, 1986). The actived form with bound cofactor is the holoenzyme of GAD (holoGAD), while the inactive form without bound cofactor is the apoenzyme of GAD (apoGAD). ApoGAD can account for more than 50% of neuronal GAD and can serve as a reservoir of enzyme when additional GABA production is needed (Itoh and Uchimura, 1981). Again, GAD_{65} seems to account for the great majority of apoGAD, but GAD_{67} also contributes to the pool (18%) (Martin *et al.*, 1991). Experimentally, determination of apoGAD and holoGAD concentrations is difficult because of enzyme intraconversion. Quantification of GAD_{65} and GAD_{67} making up the apoenzyme and holoenzyme pools cannot be determined by enzyme activity because both GAD isoenzymes contribute to both pools.

After repeated cocaine, there is a significant decrease in striatal endogenous GABA release and trend towards a decline in tissue GABA (see Figure 3-3 and 3-4). Since endogenous GABA release is coupled to GAD activity (Tapia, 1983), I hypothesized that changes in GAD activity was a possible mechanism for decreased endogenous GABA release and total GABA available for release. Previously, Lindefors reported that there was decreased GAD_{67} mRNA in the nucleus accumbens after repeated amphetamine in rats (Lindefors, 1993). If this result is extended to repeated cocaine, then this could potentially be a long term change in regulation of GABA production. There is no change in GAD_{67} mRNA levels in the striatum of cocaine-sensitized rats (Sorg *et al.*, 1995). Since, the functional activity of GAD cannot be determined by changes in the amount of GAD mRNA levels, it is more important to determine both GAD activity after cocaine sensitization and in the presence of cocaine.

In this study, a GAD activity assay was developed to measure GABA production. Next, GAD activity was examined after cocaine sensitization (*in vivo*) and in the presence of cocaine (*in vitro*). The results show that neither cocaine sensitization nor *in vivo* cocaine impairs the ability of GAD to produce GABA.

Methods

Animals and Treatment

Male Sprague-Dawley rats were used and housed as described in Chapter 2. Naive rats were used to characterize the GAD assay. For cocaine treated rats, the drug treatment regimen started after three daily saline injections (1.0 ml kg^{-1} i.p.) in the home cages. In two sets of experiments, animals received either 1.0 ml kg^{-1} saline i.p. (Group S, N = 6), or cocaine (15 mg kg^{-1}, i.p., N = 6). Rats were killed 24 h after their last injection to obtain striatal tissue. Striatum was stored at -80°C until analysis.

Behavioral Rating

Behavior was rated as described in Chapter 2 on Days 1, 8 and 14. Behavior was rated for 10 sec every 2.5 min for 60 min. Rats were determined to be sensitized if there was a significant increase in behavioral scores after Day 1.

Characterization of GAD Activity

Striata from cocaine naive rats were homogenized (Brinkman Polytron) and suspended in a 0.5 M HEPES (pH 7.0) buffer with 10 mM 2-aminoethyl-isothiouronium bromide, 200 μM pyridoxal phosphate, 20 μM gabaculine, and 0.5% Triton X-100 (Buffer A). To determine which protein concentration to use, striatal homogenate (400 – 1800 μg) was incubated with 4.5 mM L-glu for 30 min at 37°C. To determine what time of incubation to use, striatal homogenate was incubated with 4.5 mM L-glu from 0 to 60 min at 37°C. To determine the v_{max} of the enzyme, striatal homogenate was incubated with L-glu (1 – 20 mM) for 30 min at 37°C. The enzyme reaction was stopped by adding 10% TCA to the sample. Supernatant was collected after centrifugation for 20 min and stored at 4°C until analysis for GABA by HPLC-EC.

GAD Activity in Naive and Treated Rats

The effect of cocaine on striatal GAD activity was determined both after *in vivo* cocaine and in the presence of cocaine (*in vitro*). For the *in vivo* cocaine GAD activity assay, striata from treated rats were homogenized in Buffer A. Next, striatal homogenate was added to Buffer A with a final concentration of either 4.5 or 15 μM L-glu. For measuring the effect of *in vitro* cocaine, cocaine (1 – 1000 μM) was added with Buffer

A before incubation. Homogenate was then allowed to incubate for 30 min at 37°C. TCA was added at the end of the incubation to end the assay. For tissue blanks, TCA was added prior to incubation. Samples were then centrifuged two separate times at 4000 x *g* for 20 min. Supernatant was decanted and stored at 4°C until evaluation for GABA by HPLC-EC.

Determination of GABA by HPLC-EC

GABA was quantified by HPLC-EC (see Chapter 2 for details). Briefly, collected supernatant was resuspended in mobile phase and derivitized with o-pthalaldehyde/ mercaptoethanol reagent for 3 min prior to injection into the HPLC system. The HPLC-EC system consisted of a PM-11 Solvent Delivery System with a 50 µL sample loop and Ranin Microsorb-MV C18 column (3µm, 4.6mm x 10 cm). Electrochemical detection was accomplished by LC-4B detector with TL-5 glassy carbon electrode and recorded on HP-3390A Integrator. GABA mobile phase consisted of 0.08 M NaH_2PO_4, 0.269 mM Na_2EDTA, tetrohydrofuran 12.0%, v/v, acetonitrile 12.0%, v/v, and 2.0% propanol-1 at a pH of pH 5.6. Flow rate was 0.97 ml/min.

Protein Concentration in Homogenates

Protein concentrations of the homogenates used for GABA production were determined by using a protein-dye binding assay (Bradford, 1976).

Data Analysis

Results are expressed as mean values ± SEM; N indicates the number of animals used. The sums of the rankings for each rat from behavioral experiments on the first and last days were analyzed by the Wilcoxon signed-rank test. GAD activity was analyzed by student's *t*-test. The results were considered significant if $p < 0.05$.

Results

Characterization of GAD Activity

The GAD activity assay employed showed increases in GABA production from the striatum of naive rats that were dependent on protein concentration (Figure 4-1), incubation time (Figure 4-2), and substrate concentration (Figure 4-3). When striatal protein concentration was increased (400 – 1700 μg protein) and the concentration of GLU remained fixed (4.5 mM), the amount of GABA produced was increased in a linear fashion as concentration of protein increased (Figure 4-1). When the incubation time was increased (0 – 60 min) and the concentration of GLU remained fixed (4.5 mM), the amount of GABA produced increased in a linear fashion until the 45 min time point, where the amount of GABA produced appeared to start to plateau (Figure 4-2). The effect of changing the concentration of GLU (1 – 20 mM), but keeping time and protein constant, resulted in a rhomboidal-shaped curve, i.e. there was a steep rise from 1 mM to 10 mM, and then a plateau to 20 mM (Figure 4-3). When GABA production was transformed to a Lineweaver-Burke plot, the v_{max} was 970 nmol h^{-1} and the K_m was 3.09 mM (Figure 4-4). Based on these results, an incubation time of 30 min and GLU concentration of 4.5 and 15 mM were chosen to test GAD activity from the striatum of saline and cocaine treated rats.

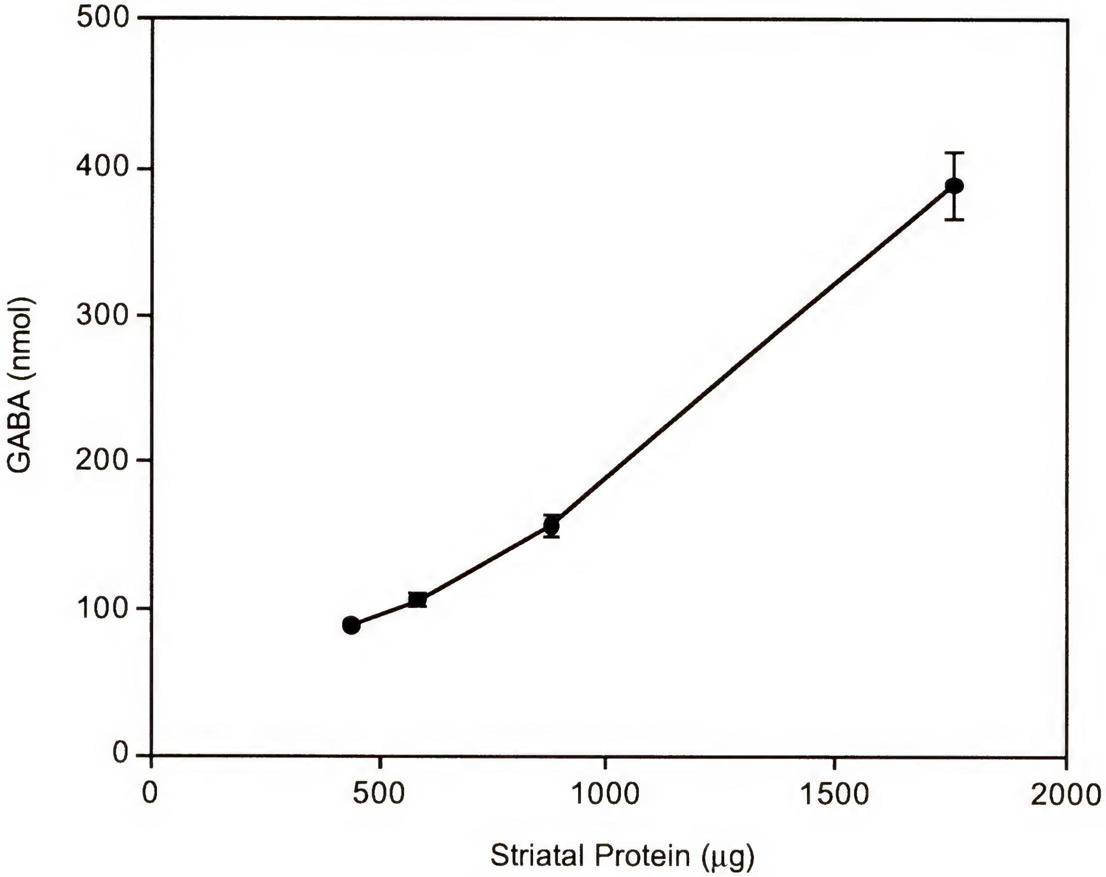

Figure 4-1 GABA production is dependent on the amount of striatal protein. Different concentrations of striatal protein (400 – 1800 μg) were incubated with glutamic acid (4.5 mM) to produce GABA. GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM. For each point, the experiment was done in triplicate from proteins of a pooled striatal homogenate. For some points, the error was smaller than the point.

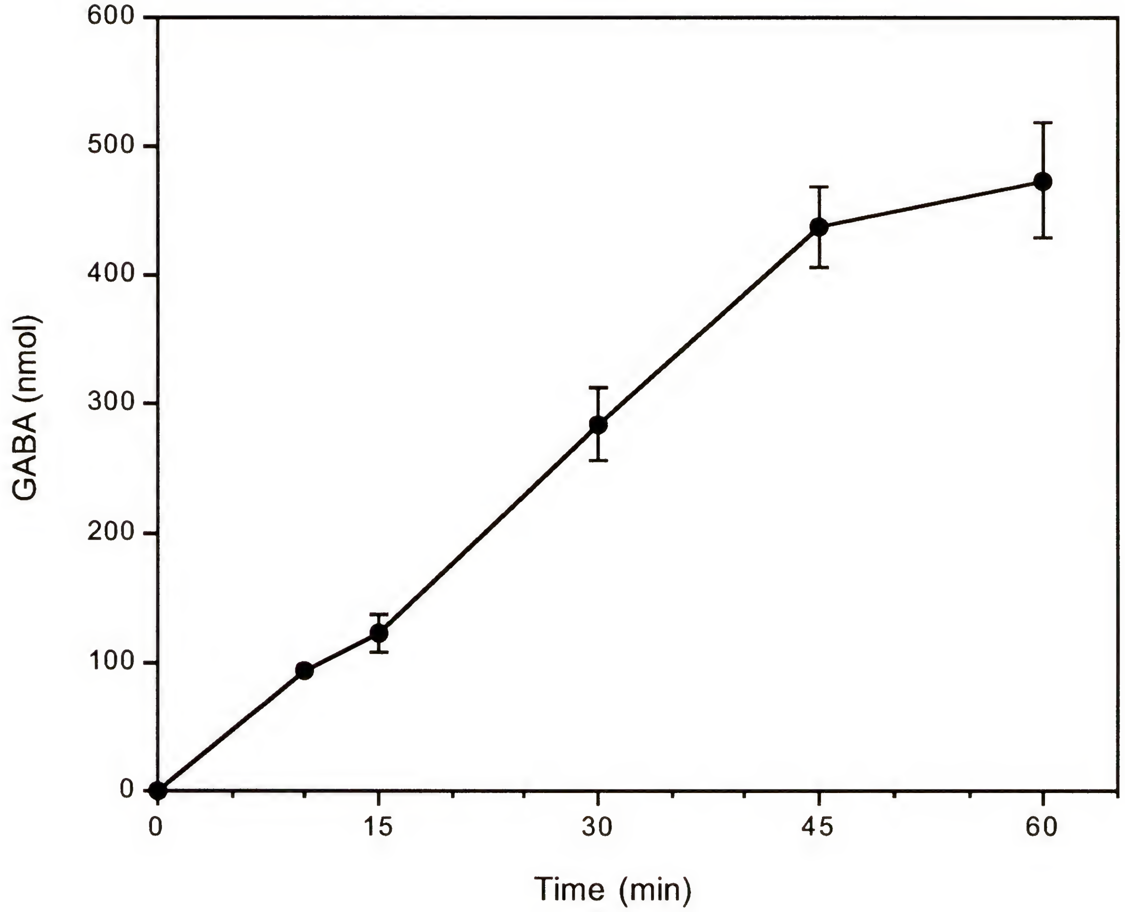

Figure 4-2 GABA production is dependent on the incubation time. GABA production from glutamic acid by striatal tissue was measured at various times (0 – 60 min). GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM. For each point, the experiment was done in triplicate. For some points, the error was smaller than the point.

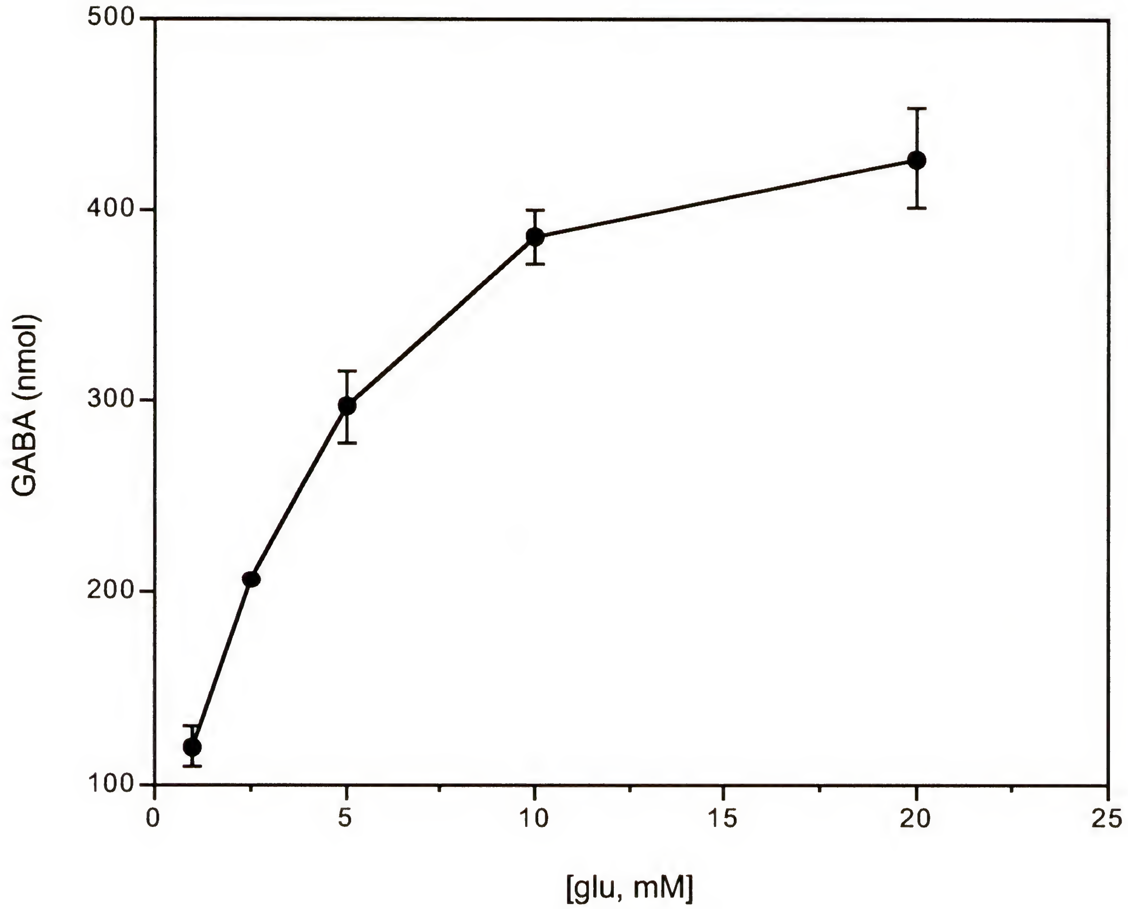

Figure 4-3 GABA production is dependent on the concentration of glutamic acid. GABA production from glutamic acid by striatal tissue was measured at various concentrations of glutamic acid (1 – 20 mM). GABA was measured by HPLC-EC. Data are expressed as the mean ± SEM. For each point, the experiment was done in triplicate. For some points, the error was smaller than the point.

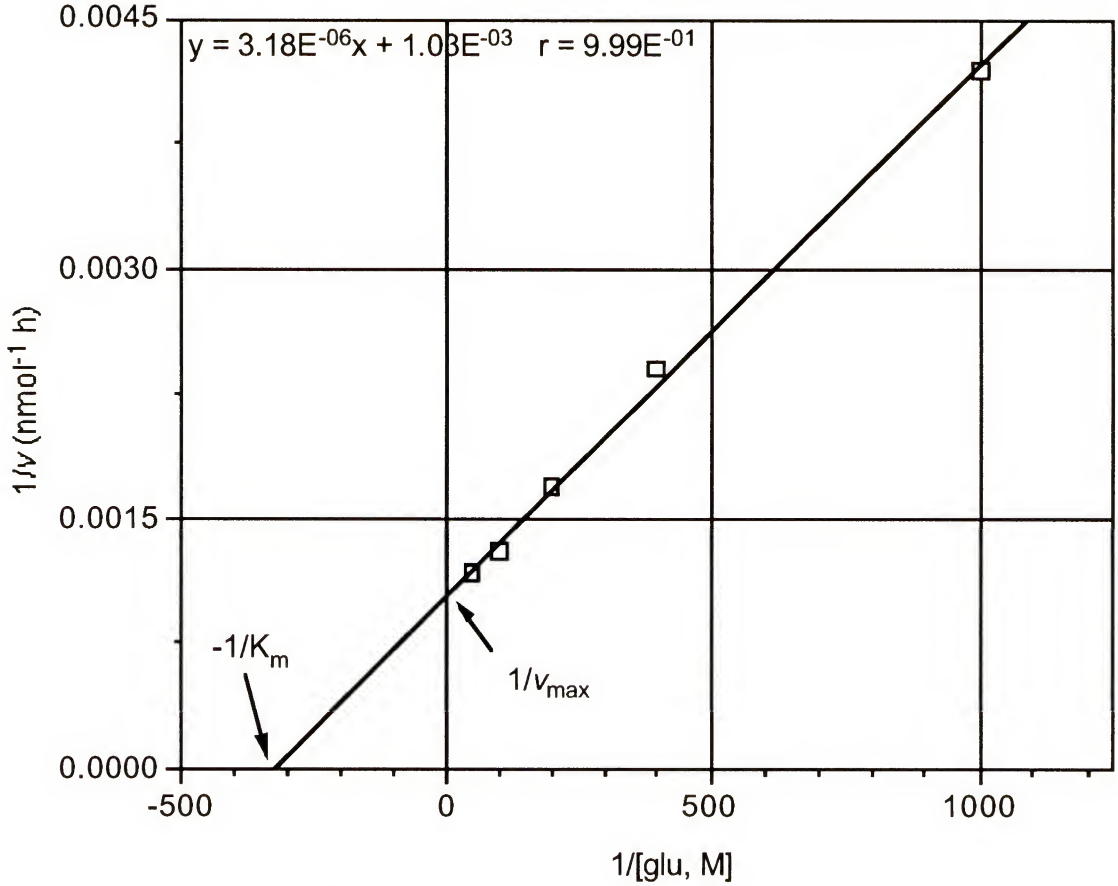

Figure 4-4. Lineweaver-Burke transformation of GABA production from Figure 4-3.

Behavior

Rats were treated with either repeated saline or cocaine injections. Rat behavior was rated on Day 1, 8, and 14 (Figure 4-5). Group S treatment did not result in a significant change in behavior across days. However, Group C15 treatment resulted in a significant effect of cocaine across days. When Group C15 treatment was compared across days, cocaine Day 8 was significantly different than cocaine Day 1 ($p < 0.05$). In this study, only four of the six cocaine treated rats showed higher behavioral scores when Day 14 is compared to Day 1, but five of the six cocaine treated rats showed higher behavioral score on Day 8 compared to Day 1 (data not shown). In addition, two of the six cocaine treated rats on Day 1 had behavioral scores that were equal to (69 vs.

69) or close to (59 vs. 65) their Day 8 behavioral scores, but were lower than their Day 14 behavioral scores (data not shown).

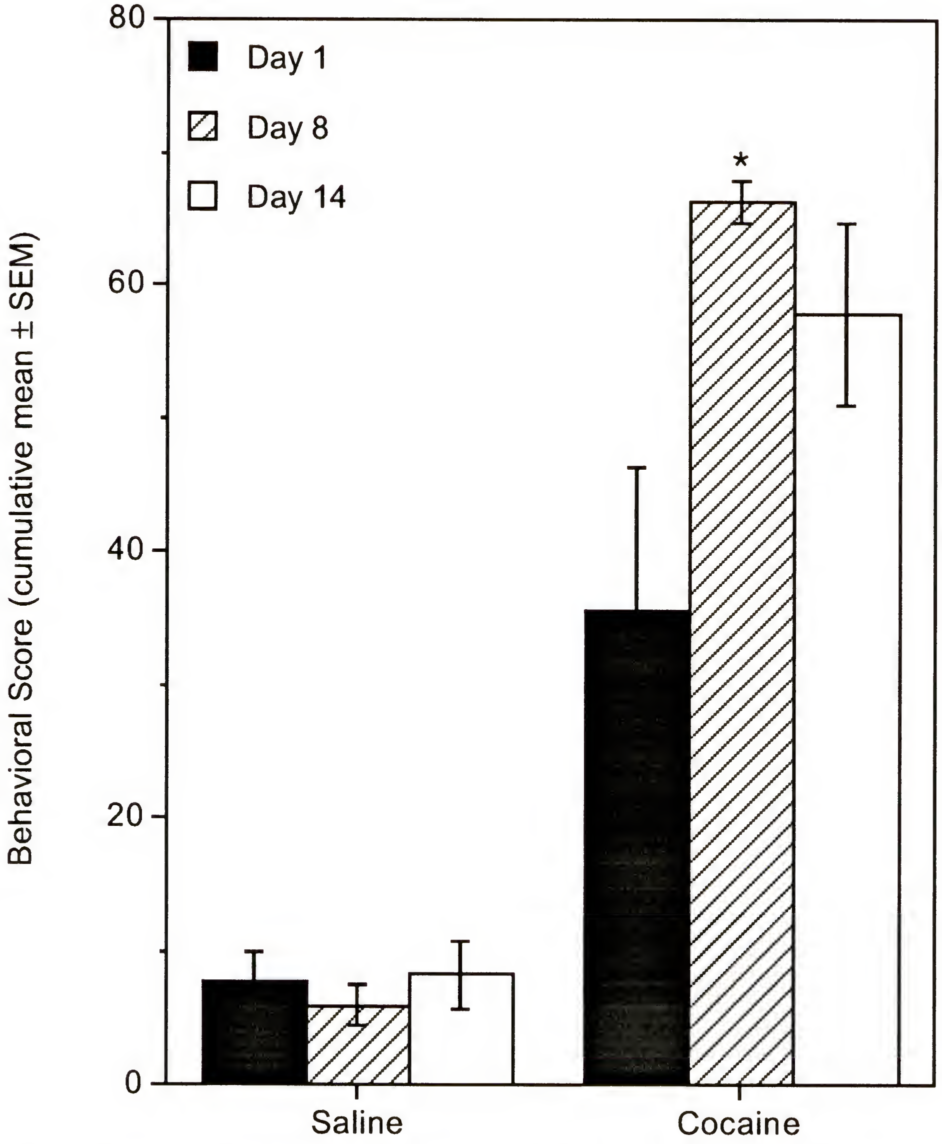

Figure 4-5 Cumulative behavioral scores of saline (0.1 ml kg^{-1}) and cocaine (15 mg kg^{-1}) treated rats on Day 1, 8 and 14. Behavior was rated for 10 sec every 2.5 min for 60 min. Daily cocaine treatment led to significantly greater behavior on Day 8 compared to Day 1 ($p < 0.05$). The cumulative behavioral scores within treatment groups were compared across days using the Wilcoxon signed-rank test. Data are expressed as the cumulative mean ± SEM for N = 6 per group. * $p < 0.05$

GAD Activity after *In Vivo* Treatment

GAD activity was measured from the striatum of Group S and Group C15 rats. GABA production was measured after 30 min of incubation with one of two glutamate concentrations equivalent to either a saturating concentration (15 mM) or the approximate K_m (4.5 mM) for GAD (Figure 4-6). There was no difference in GABA production at a saturating concentration of glutamate between Group S and Group C15. Neither were there differences at the approximate K_m concentration of GAD between Group S and Group C15. Therefore, cocaine sensitization did not result in decreased GAD activity.

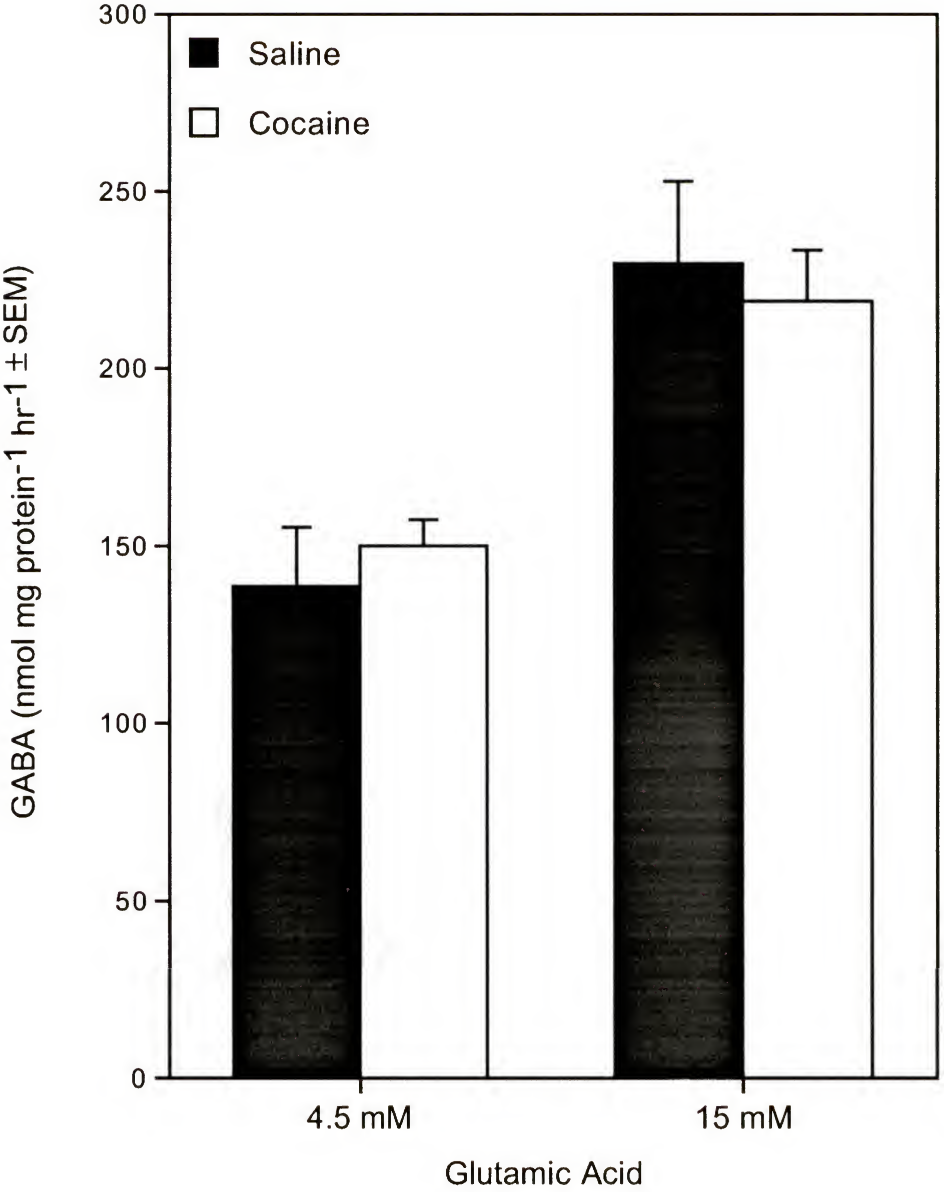

Figure 4-6 The effects of repeated cocaine on striatal GAD activity. GAD activity was measured from the striatum of rats treated with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) once daily for 14 days. GABA formation in the presence of 4.5 or 15 mM glutamate was measured by HPLC-EC. Data are expressed as the mean ± SEM for N = 6.

GAD Activity after *In Vitro* Cocaine

The direct effect of *in vitro* cocaine (1 – 1000 µM) on GAD activity was also measured (Figure 4-7). Cocaine did not affect GAD activity by more than 5% in either direction (95 to 104.8% of control) when GABA production was measured at a saturating concentration of glutamate (15 mM).

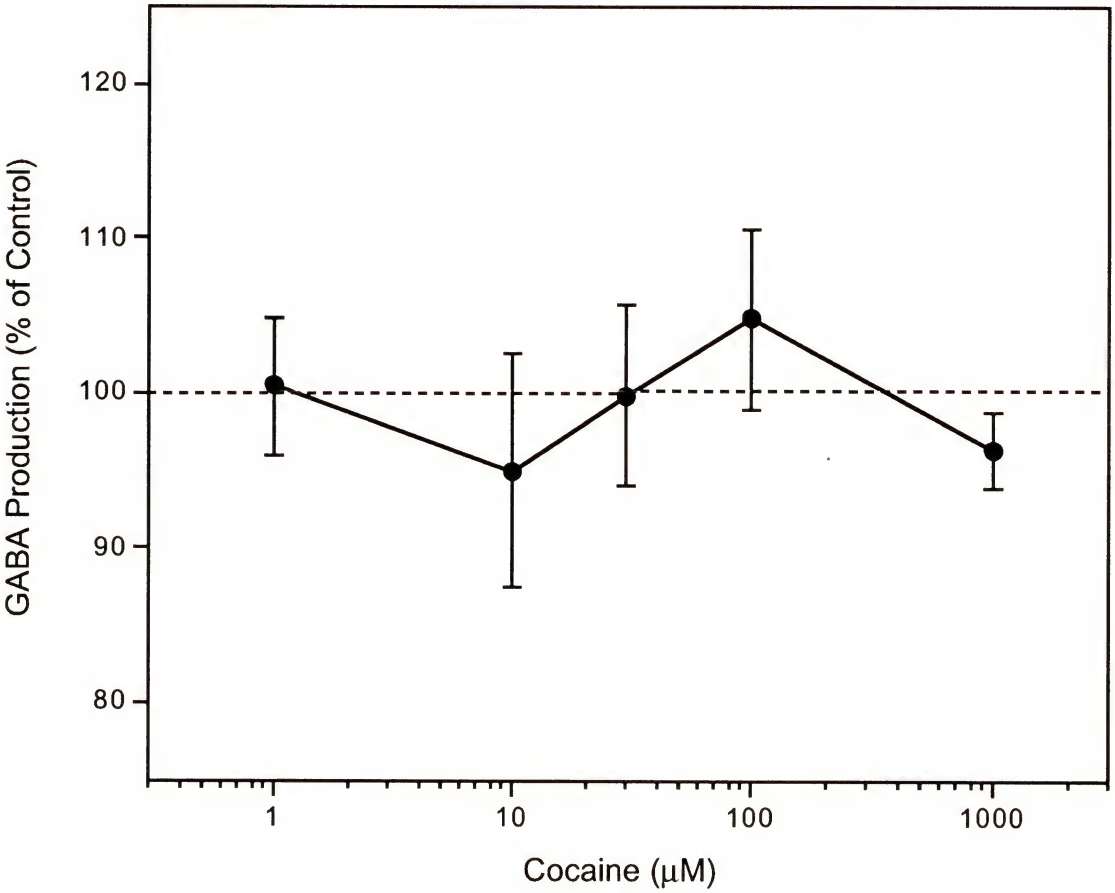

Figure 4-7 The effect of *in vitro* cocaine on striatal GAD activity. GAD activity was measured in the presence of different cocaine concentrations (1 – 1000 µM) with striatal brain homogenate of cocaine naive rats. GABA production was measured by HPLC-EC. Data are expressed as the mean ± SEM for N = 3 – 5 experiments done in triplicate.

Discussion

After repeated cocaine, there was a significant decrease in striatal endogenous GABA release and a trend towards a decline in tissue GABA (see Chapter 3). Since GAD activity both regulates GABA production and is coupled to endogenous GABA release (Tapia, 1983), changes in GAD activity could be a mechanism for decreases in endogenous GABA release after cocaine sensitization. *In vivo* cocaine could decrease the amount of GABA produced by GAD through the reduction of enzyme levels or a direct inhibition of enzyme activity. In this study, GAD activity was examined after cocaine sensitization (*in vivo*) and in the presence of cocaine (*in vitro*).

First, appropriate conditions for the GAD activity assay were determined. The GAD assay produced GABA in a manner typical of an enzymatic process. Striatal GABA production was dependent upon protein concentration, incubation time and glutamate concentration. GAD activity for the striatum was within the range of previously reported results (Löscher *et al.*, 1989). The reported K_m of glutamate for GAD from the literature ranges from 0.1 to 1.0 mM (Martin and Rimvall, 1993), while we determined the K_m in this study to be 3.09 mM. Concentrations of glutamate below 1.0 mM were not tested because of an oversight on my part; consequently, there may have been an error in determination of the K_m.

When rat behavior was measured, Group C15 rats had significantly higher behavior on Day 8 compared to Day 1, but not on Day 14 compared to Day 1. The difference could be explained by the high behavioral scores on Day 1 of two of the six rats of Group C15. Both of these rats had more than 50% of their scores at 3, which normally occurs only after sensitization. On Day 8, Group C15 rats had higher or equal behavioral scores compared to Day 1 that occurred within a narrow range (61-70). This effect was not unexpected because it has been shown previously that cocaine sensitization can occur only after eight days of cocaine treatment (Peris *et al.*, 1990). However, nearly 75% of the scores were 3, which explains both the high and narrow range of behavioral score. Finally on Day 14, Group C15 had four of six rats with higher scores than Day 1, but this was less than Group C15 Day 8 rats (5 of 6). The two rats from Group C15 having high behavioral scores on Day 1 were the rats that did not show an increase in behavioral score on Day 14. The expected increase in behavioral score when Day 14 is compared to Day 1 may be due to the previously mentioned rats from Day 1. Their behavior on Day 1 was higher than expected and added to the variability in behavior. The variability of behaviors of Group C15 on Day 8 and 14 are within the same range seen in the following chapters. However, since the cocaine treated rats did not show behavioral sensitization on Day 14, the effects of sensitization may not be present in the assays measuring GABA production with treated striatal homogenates.

When striatal GAD activity was measured from treated rats, there were no differences in GAD activity between saline and cocaine treated rats at the approximately determined K_m concentration of glutamate for GAD. As stated before, the estimated K_m for this study may be high and as a result may be an inaccurate measure of changes in affinity of GAD for glutamate. However, if there were a change in GAD affinity for glutamate, then one should expect at minimum a significant shift in the production curve of GABA that should affect both the K_m concentration and any other concentration below saturation, but above 20% of minimal GABA production. Since GAD activity was not changed between saline and cocaine treated rats at 4.5 mM, it is difficult to believe that GABA production would change at the true K_m of GAD and thus represent a change in the affinity of GAD for glutamate. It has been previously reported in rats self administering cocaine that GABA turnover was increased after withdrawal from cocaine (Dworkin *et al.*, 1995). Thus, the rate of GABA synthesis would be expected to increase after repeated cocaine. However, it could also be possible that the rate of GABA uptake or metabolism was increased. Since in this study GABA-T activity was blocked by gabaculine, it may be important in future experiments to measure the effect of repeated cocaine on GABA metabolism.

The ability of GAD to synthesize GABA at a saturating concentration of glutamate represents the maximal ability of the enzyme and provides a simple measure of functional GAD. It would be expected that if there were reduced GAD to produce GABA, then GABA would be produced at a lower rate and in lesser total amount. In this study, cocaine sensitization did not change the ability of GAD to produce GABA from a saturating concentration of glutamate. These results support the lack of changes in GAD_{67} mRNA levels in the striatum of cocaine-sensitized rats (Sorg *et al.*, 1995). However, it has been shown that GAD_{67} mRNA is decreased in the nucleus accumbens after repeated amphetamine in rats (Lindefors *et al.*, 1992). Both measurements of GABA production at the saturating concentrations and the K_m of glutamate would be insensitive to selective changes in the amounts of GAD_{65} and GAD_{67}, therefore it may be important to determine whether specific changes occur in either GAD_{65} or GAD_{67} expression after repeated exposure to cocaine. In a future experiment, both striatal GAD_{65} and GAD_{67} proteins should be examined after cocaine sensitization to determine this possibility.

When cocaine was added *in vitro* to the GAD activity assay with striata of naive rats, it did not significantly change the ability of GAD to produce GABA. Potentially, brief and limited exposure of GAD to cocaine repeatedly could change the overall activity level of GAD. Our results indicate that this is a less likely scenario, but a direct measurement of the effect of *in vitro* cocaine on GAD activity from sensitized rats would be important especially if activity of the isoenzymes could be separated possibly by specific GAD isoenzyme inhibitors. If *in vitro* cocaine had a specific effect on a GAD isoenzyme activity, then this could result in different amounts of GABA in the synaptosome or metabolic GABA compartments.

The regulation of GAD activity is complex (Martin and Rimvall, 1993). In addition to two GAD isoenzymes, GAD exists in two activity states; holoenzyme (active) and apoenzyme (inactive). Less is known about long term regulation of GABA synthesis and the consequence of changes in GAD message levels and protein expression. Other regulators of GAD activity include the cell's energy status, GABA levels, and pyridoxal phosphate levels. The effect of repeated cocaine on these regulators of GAD activity should be considered in future experiments. In addition, eliminating the action of repeated cocaine on GAD activity may be premature considering the difficulty in separating the contribution of the isoenzymes to GABA available for release. Nevertheless in this study, repeated cocaine did not decrease the ability of striatal GAD to synthesize GABA. In addition, *in vitro* cocaine did not change striatal GAD activity.

CHAPTER 5
FUNCTIONAL MODULATION OF GABA$_A$ RECEPTOR BINDING AFTER COCAINE SENSITIZATION

Introduction

The GABA$_A$ receptor is important because it contains ligand binding sites that are clinically relevant such as the BZD and anti-convulsant sites (Sieghart, 1992). The specific GABA$_A$ receptor agonist, muscimol, binds to the GABA recognition site on the ß subunit of the GABA$_A$ receptor to stimulate receptor function (Zezula *et al.*, 1996). Flunitrazepam and clonazepam bind to the BZD site on the α subunit of the GABA$_A$ receptor and allosterically modulate receptor function (Sieghart, 1995). The convulsant site is located near the chloride ionophore on the GABA$_A$ receptor and can bind TBPS (Olsen *et al.*, 1990). After repeated cocaine, there are reported increases in BZD binding (Goeders, 1991) and decreases in convulsant binding (Pecins-Thompson and Peris, 1993). Although there were differences in procedures involving length of repeated cocaine and withdrawal, the major difference between these studies was that different non-competitive sites on the GABA$_A$ receptor were measured. Since the main GABA binding site on the GABA$_A$ receptor was not measured and this site interacts with the BZD binding site, the effect of repeated cocaine on these binding sites was examined in this study.

Functional binding can be used to describe two experimentally different results; (1) receptor binding that can be changed by other ligands such as allosteric modulators (Sieghart, 1992), and (2) receptor binding that occurs at physiologic relevant concentrations (Edgar and Schwartz, 1992). Although both descriptions are highly informative, the first description is more commonly used to describe the GABA$_A$ receptor because of the allosteric ligand sites found on the receptor and will also be used in this chapter. Allosteric ligand sites confer additional regulatory control on receptor function. In the case of the $GABA_A$ receptor, the effect of allosteric modulators on receptor function can be measured both by changes in receptor binding and changes in agonist stimulated $^{36}Cl^-$ uptake. This chapter will examine functional binding after repeated cocaine, while the next chapter will examine changes in stimulated $^{36}Cl^-$ uptake.

Functional binding is affected by a variety of receptor changes. First, changes in the affinity of the receptor for the ligand at either the main ligand binding site or the allosteric ligand binding site could affect functional binding. Second, changes in the number of allosteric binding sites, but not the main ligand binding site could indicate allosteric receptor decoupling (Roca *et al.*, 1990). After repeated cocaine, the increase in BZD binding (Goeders, 1991) and decrease in convulsant binding (Pecins-Thompson and Peris, 1993) could be explained if the $GABA_A$ receptor ionophore is decoupled from the allosteric BZD site. This could occur if the composition of the $GABA_A$ receptor subunits were changed after sensitization. Since GABA stimulated $^{36}Cl^-$ uptake was decreased in cocaine-sensitized rats (Peris, 1996), we hypothesized that the functional binding sites of the $GABA_A$ receptor were decoupled after cocaine sensitization.

The binding of [^3H]-muscimol has not been characterized previously in this laboratory. In this study, homogenate binding was performed on cerebellar tissue because it contains high concentrations of $GABA_A$ receptor (Beumont *et al.*, 1978; Palacios *et al.*, 1981) and cerebellar tissue was readily available. For QAR, the region of primary interest is the STR because of the hypothesized effects of repeated cocaine on this brain area. In addition, the STR has regional differences between its head and tail regions, thus the STR was measured at two levels of the brain. The CTX was measured because $GABA_A$ receptor composition in this brain area is different and *in vivo* cocaine does not affect this brain area. NAc was also measured because it would be interesting to determine if changes in $GABA_A$ receptor binding could be related to cocaine addiction.

In this study, binding to the GABA site and functional binding by allosteric modulators were measured after repeated cocaine. Since the previous reports did not measure the status of the main GABA binding site after repeated cocaine, it would be informative to evaluate the main regulatory site on the $GABA_A$ receptor. Previous reports on $GABA_A$ receptor number also did not evaluate the $GABA_A$ receptor's ligand binding ability to be functionally modulated after repeated cocaine, which could be relevant if receptor binding was decoupled. The results obtained after cocaine sensitization showed no change in the GABA site and, contrary to previous results, no change in BZD binding. In addition, $GABA_A$ receptor functional binding was not changed by repeated cocaine as indicated by flunitrazepam modulation of $[^3H]$-muscimol or GABA modulation of $[^3H]$-flunitrazepam binding.

Methods

Animals and Treatment

Male Sprague-Dawley rats were used and housed as described in Chapter 2. Naive rats were used to characterize $[^3H]$-muscimol binding. For cocaine sensitization, the rats were treated as follows. After two or three daily saline injections (1.0 ml kg^{-1} i.p.) in the home cages, the drug treatment regimen started. Rats received either 1.0 ml kg^{-1} saline i.p. (Group S, N = 11) or 15 mg kg^{-1} cocaine i.p. (Group C15, N = 11). In this study, two separate groups of treated rats were prepared at separate times. Rats were killed 24 h after their last injection to obtain brain hemispheres. Right and left brain hemispheres were rapidly frozen with powdered dry ice until brain slicing. Brains were stored at -80°C until sectioning which was between three and five months.

Behavioral Rating

Behavior was measured as described in Chapter 2. Behavior was rated for 10 sec every 2.5 min for 60 min. Rats were determined to be sensitized if there was a significant increase in behavioral scores when Day 14 was compared to Day 1.

Characterization of [^{3}H]-Muscimol Binding

[^{3}H]-Muscimol binding was performed based on a protocol by Beumont and colleagues (Beumont *et al.*, 1978). Briefly, cerebellum from naive rats were homogenized in a Potter-Elvehjam tissue grinder (Wheaton) in 15 ml of 0.31M Tris-citrate buffer (pH 7.1, TC buffer). After homogenization, tissue was centrifuged at 900 x *g* for 10 min in a Sorvall SA600 rotor at 4°C. The resulting pellet was discarded, while the supernatant was again centrifuged at 20,000 x *g* for 20 min. The pellet was resuspended in TC buffer and homogenized. The homogenate and the pellet was centrifuged twice more, once at 8,000 x *g* for 20 min and then 20,000 x *g* for 20 min. The supernatant was discarded and the pellet was frozen at -15°C until the binding assay was performed. For the final centrifugation, the pellet was resuspended in 50 mL of TC buffer and centrifuged at 20,000 x *g* for 20 min for a total of three times. The resulting pellet was used for the homogenate binding assays.

For determining optimal protein concentration, [^{3}H]-muscimol (78 – 104 nM, 12.05 Ci mmol^{-1}, NEN Life Science Products) was incubated with varying concentrations of cerebellum protein (2 – 220 µg) isolated from the preceding procedure at 4°C for 60 min in TC buffer. TC buffer was used for the following experiments unless stated otherwise. Non specific binding was determined by GABA (100 µM). Samples were carried out in duplicate. At the end of incubation, the tissue was filtered onto glass fiber filters (Whatman) and washed twice with 4 mL of fresh TC buffer. The amount of [^{3}H]-muscimol retained on the filters was determined by liquid scintillation. Specific

$[^3H]$-muscimol binding was defined as the difference between the total amount of $[^3H]$-muscimol binding minus the amount of $[^3H]$-muscimol binding with GABA added.

For the competitive binding assay, $[^3H]$-muscimol (79 – 90 nM, 12.05 Ci $mmol^{-1}$, NEN Life Sciences) was incubated with various concentrations of GABA (0.003 – 1000 μM) at 4°C for 60 min. At the end of incubation, the tissue was filtered and counted as described above.

$[^3H]$-Muscimol Binding

Brains were cut into 15 μm sections and mounted onto slides as described in Chapter 2. Two regions of the brain were identified and isolated according to coordinates by Paxinos and Watson (Paxinos and Watson, 1993). The first slice contained the head of the STR, CTX and NAc (rostral). The second slice contained the body-tail of the STR and the CTX (caudal). Slide mounted sections were brought to room temperature and allowed to completely dry. TC buffer was used for the following experiments unless stated otherwise. The wash incubation consisted of two 20 min incubations at 4°C. 30 nM $[^3H]$-muscimol binding was for 90 min at 4°C. Non-specific $[^3H]$-muscimol binding was determined by GABA (200 μM). On adjacent sections in a separate experiment, 30 nM $[^3H]$-muscimol was co-incubated with 1 μM flunitrazepam for 90 min at 4°C. Non-specific $[^3H]$-muscimol binding, which also contained 1 μM flunitrazepam, was determined by GABA (200 μM). The reported K_D for GABA is 360.3 nM (Bristow and Martin, 1989). After total and non-specific binding, slides were washed for 1 min at 4°C and then dipped in distilled H_20 for 1 sec. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm (Amersham) for 6 weeks. Film was developed as described in Chapter 2.

[^{3}H]-Flunitrazepam Binding

Additional adjacent slide mounted sections, previously described, were brought to room temperature and allowed to completely dry. The buffer for this experiment was Ca^{2+} buffer (see Chapter 2). The wash incubation consisted of one 20 min incubation at 4°C. The K_D for flunitrazepam has been reported to be 3.0 nM in the presence of GABA (Villar *et al.*, 1989). 3 nM [^{3}H]-Flunitrazepam (82 Ci $mmol^{-1}$, NEN Life Science Products) binding was for 30 min at 4°C. Non-specific [^{3}H]-flunitrazepam binding was determined by 3 μM clonazepam. The K_i of clonazepam for [^{3}H]-flunitrazepam binding has been reported to be 0.51 nM (Greenblatt *et al.*, 1987). On adjacent sections in a separate experiment, 3 nM [^{3}H]-flunitrazepam was co-incubated with 200 μM GABA for 30 min at 4°C. Non-specific [^{3}H]-flunitrazepam binding was also determined with both 200 μM GABA and 3 μM clonazepam. After total and non-specific binding, slides were washed in buffer for 6 min at 4°C and then dipped in distilled H_20 for 1 sec. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm (Amersham) for 4 weeks. Film was developed as described in Chapter 2.

Film Analysis

Film analysis was performed as described in Chapter 2. Briefly, the digital image of each section was captured onto a personal computer using a light box and a video camera. Brain sections were identified according to coordinates by Paxinos and Watson (Paxinos and Watson, 1993). The rostral region contained the head of the STR, CTX and NAc. The caudal region contained the body-tail of the STR and the CTX. The digital image was then analyzed with quantitative imaging software (BRS2). Film density was normalized using [^{3}H]-Microscales (Amersham) against a known concentration of protein. Specific binding was determined by subtracting non-specific binding from total binding.

Data Analysis

The data are expressed as the mean ± SEM; N = number of experiments or subjects. For statistical analysis, Wilcoxon signed-rank test was group behavior across days. Mann Whitney U was used to determine within group differences. When appropriate for binding results, ANOVA was used to analyze QAR binding. When appropriate, the Bonferroni-Dunn analysis was used as the *post-hoc* follow up test. A *p* value less than 0.05 was considered to be significant.

Results

Characterization of [^{3}H]-Muscimol Binding

The [^{3}H]-muscimol binding assay showed specific binding that was protein concentration dependent and could be displaced by GABA. When protein concentration was increased, specific [^{3}H]-muscimol binding also increased in a fairly linear fashion (Figure 5-1). Specific [^{3}H]-muscimol binding reached equilibrium 0.5 – 2 hr after incubation (data not shown). In addition, GABA displaced 100% total [^{3}H]-muscimol binding in a dose dependent manner with an approximate IC_{50} of 0.2 μM (Figure 5-2).

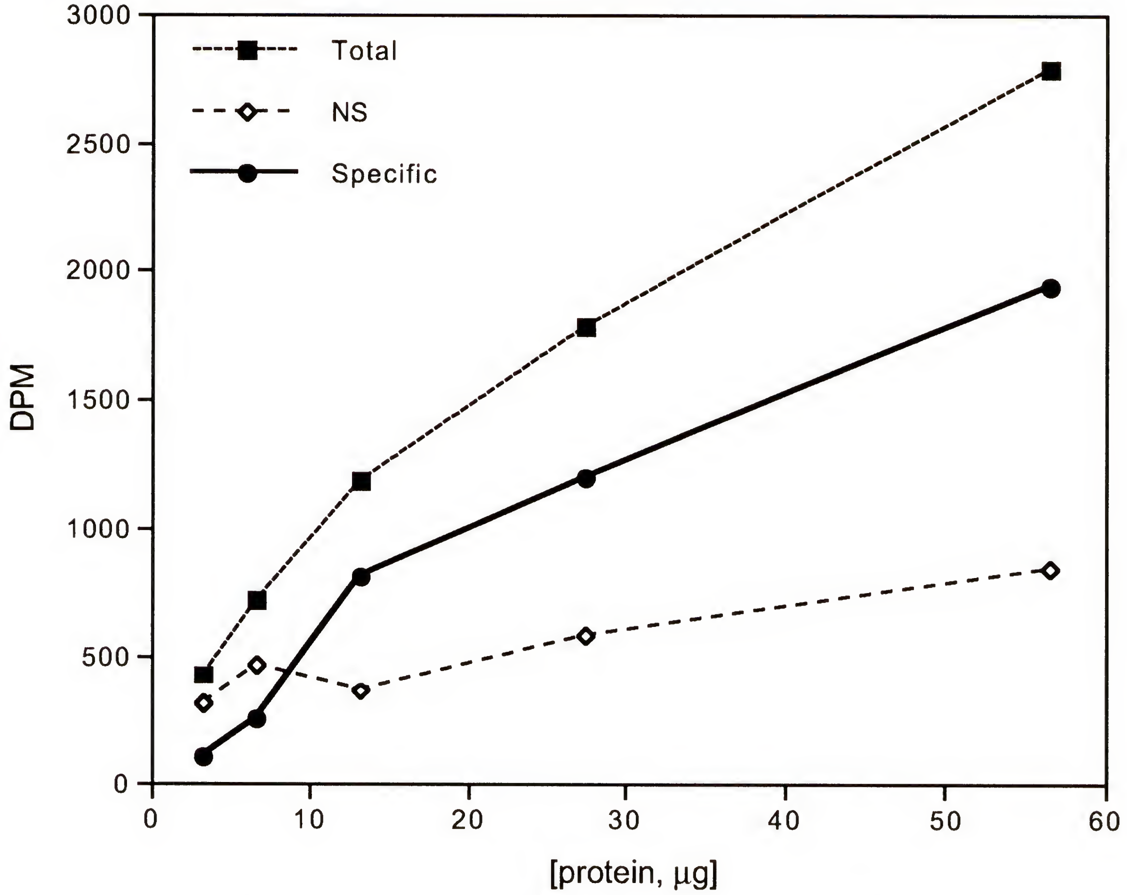

Figure 5-1 Representative [^{3}H]-muscimol binding with increasing concentrations of cerebellar protein. Specific [^{3}H]-Muscimol binding increases when protein concentration was increased. Different concentrations of protein (2 – 73 μg) were incubated with 78 nM [^{3}H]-muscimol at 4° for 1 hr. Experiment was done with duplicate points. Non-specific binding, NS

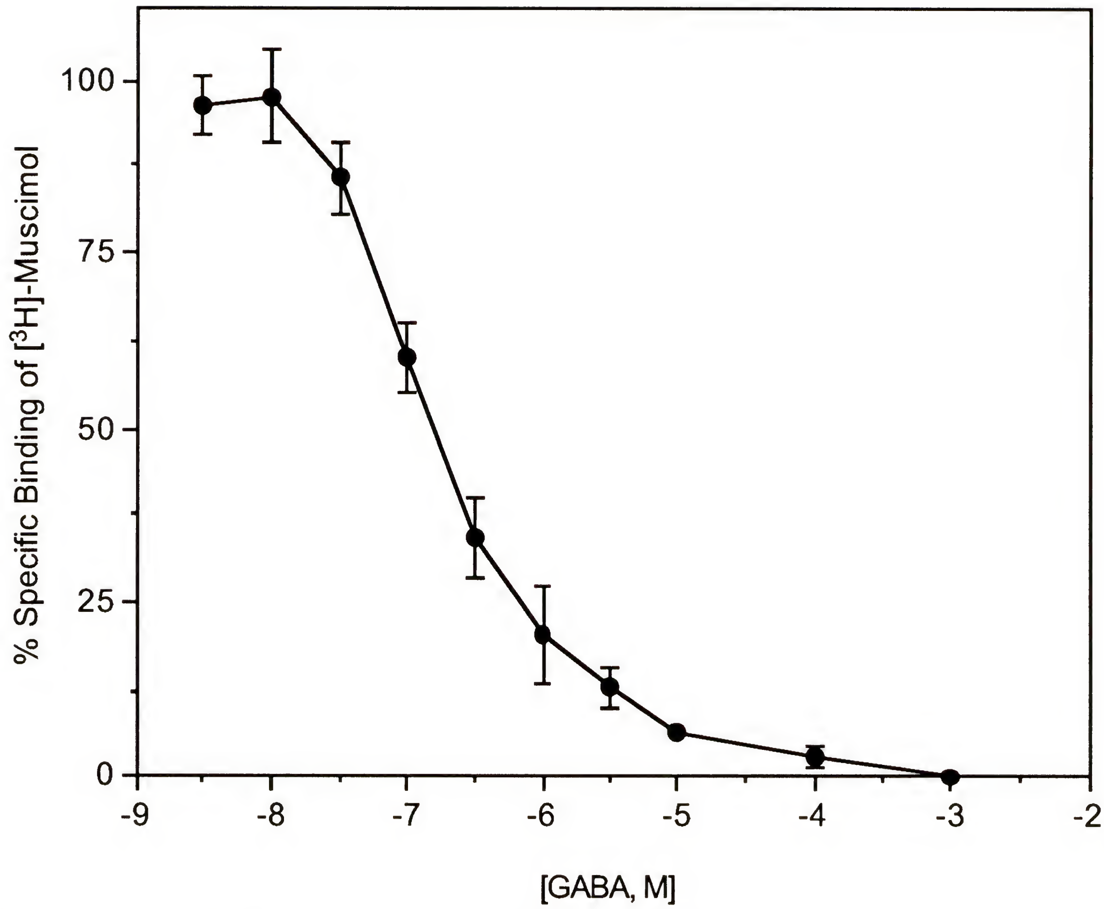

Figure 5-2 [^{3}H]-Muscimol specific binding with GABA competition in the cerebellum of naive rats. GABA (0.003 – 1000 μM) displaced [^{3}H]-muscimol specific binding in a dose dependent manner. [^{3}H]-Muscimol binding was in the presence of GABA at 4°C for 30 min. Data are expressed as the mean ± SEM for N = 3. For some points, the error was smaller than the point.

Behavior

Rats were treated with either repeated saline or cocaine injections. Rat behavior was rated on Day 1 and 14 (Figure 5-3). Group S treatment did not result in a significant change in behavior across days. However, Group C15 treatment resulted in a significant increase in behavior when Day 14 was compared to Day 1 ($p < 0.05$). In this study, two separate groups of treated rats were prepared. In the first group of cocaine treated rats, only three of the six cocaine treated rats showed higher behavioral scores when Day 14 is compared to Day 1 (data not shown). In the second group of cocaine treated rats, all rats (5) showed higher behavioral score when Day 14 is compared to Day 1 (data not shown). The behavioral scores on Day 1 and Day 14 between cocaine treated rats were not significantly different and were combined for ease of comparison.

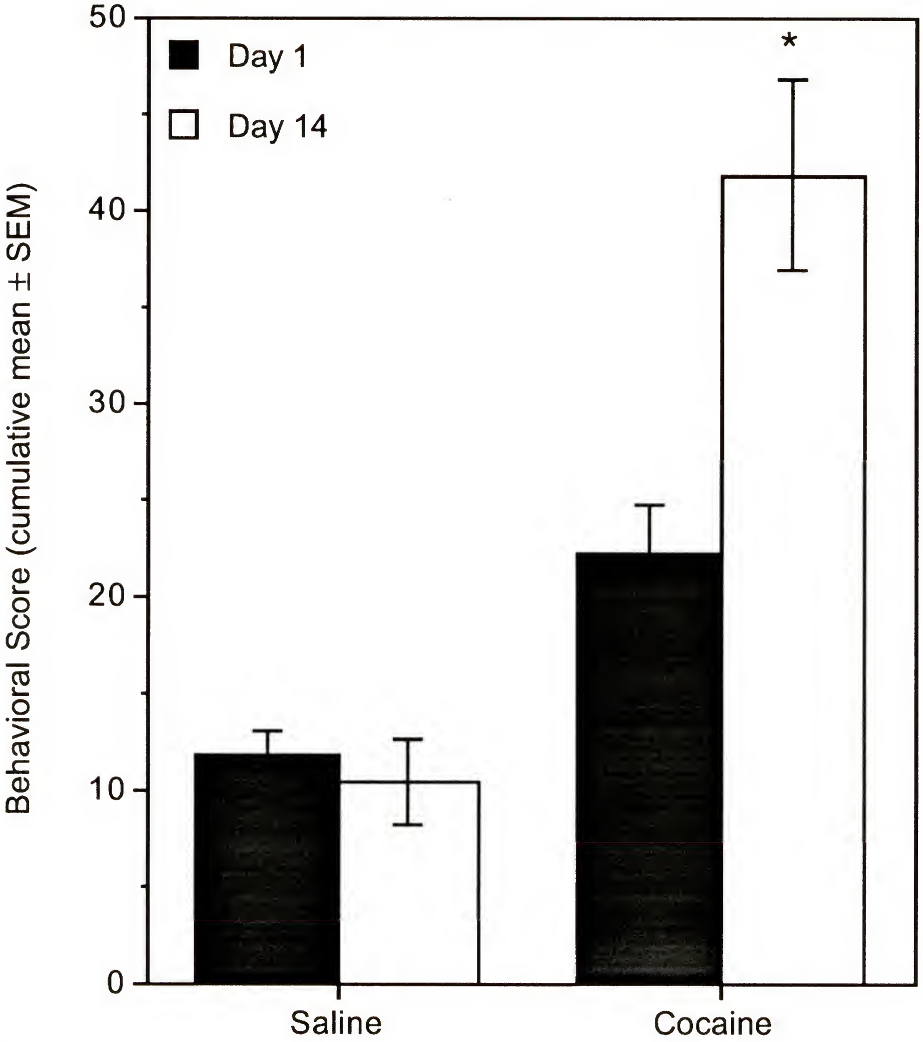

Figure 5-3 Cumulative behavior scores of saline (0.1 ml kg^{-1}) and cocaine (15 mg kg^{-1}) treated rats on Day 1 and 14. Behavior was rated for 10 sec every 2.5 min for 60 min. Daily cocaine treatment led to a significant greater behavior on Day 14 compared to Day 1 ($p < 0.05$). The cumulative behavioral scores within treatment groups were compared between Day 1 and 14 using the Wilcoxon signed-rank test. Data are expressed as the cumulative mean ± SEM for N = 11 per group. * $p < 0.05$ vs. Day 1 Cocaine

QAR of [^{3}H]-Muscimol Binding

The repeated administration of cocaine resulted in little or no effect on binding of [^{3}H]-muscimol in any of the regions measured (Figure 5-4). The cortex had increased [^{3}H]-muscimol binding relative to the STR and NAc of both saline and cocaine treated rats for both rostral slices [$F(2, 60) = 9.08, p < 0.01$] and caudal slices [$F(1, 40) = 29.32, p < 0.0001$].

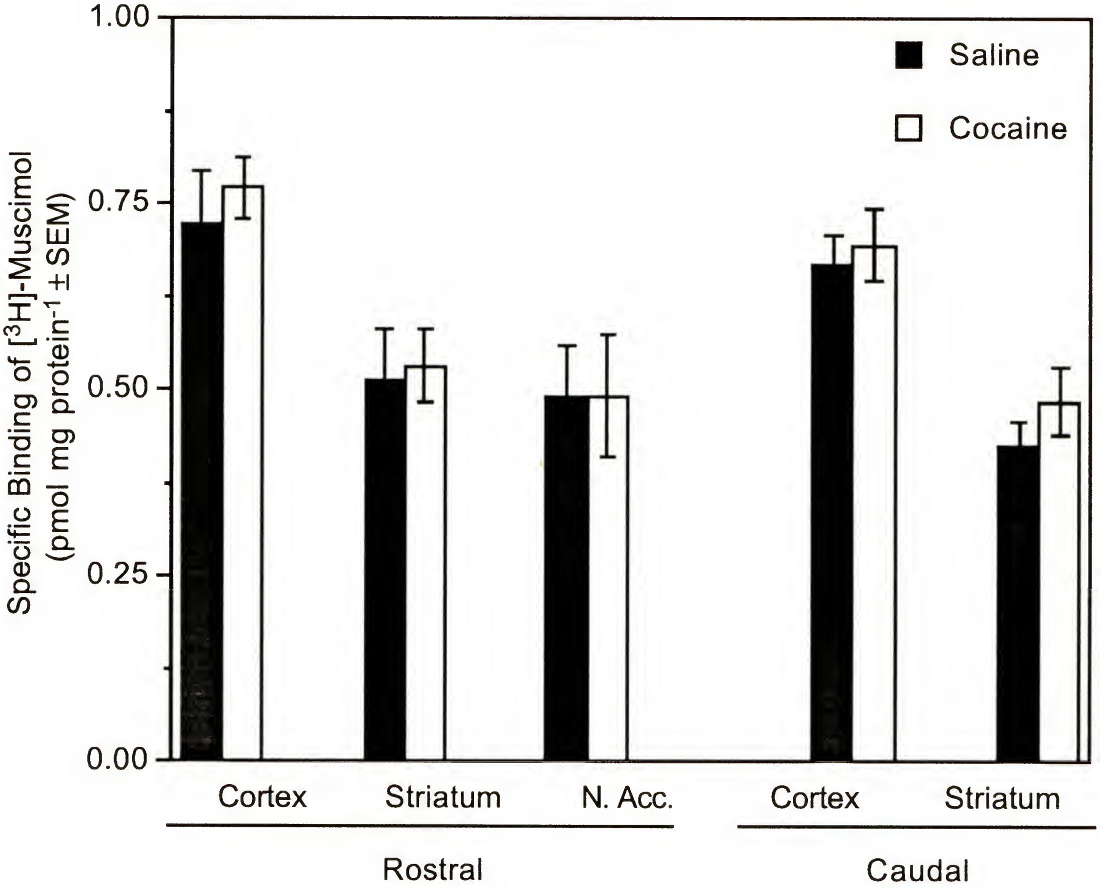

Figure 5-4 Effects of repeated cocaine on specific [^{3}H]-muscimol binding. Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 24 hr after the last treatment. There was no significant difference between the two treatment groups. The cortex in both the rostral and caudal slices had significantly increased [^{3}H]-muscimol binding (rostral, $p < 0.01$; caudal, $p < 0.0001$). Data are expressed as the mean ± SEM for N = 11.

The repeated administration of cocaine also had little to no effects on [^{3}H]-muscimol binding modulated by flunitrazepam in any of the regions measured (Figure 5-5). Again, the cortex had increased [^{3}H]-muscimol binding relative to the STR and NAc for both rostral slices [F(2, 60) = 7.49, $p < 0.01$] and caudal slices [F(1, 40) = 13.35, $p < 0.001$]. Modulation of [^{3}H]-muscimol binding with the addition of flunitrazepam was dependent on the region and treatment (Figure 5-6). In the saline treated rats, there was a trend towards enhancement of [^{3}H]-muscimol binding by flunitrazepam (23 – 53%) in all regions measured. In cocaine treated rats, the NAc showed a trend towards a decrease in enhancement of [^{3}H]-muscimol binding by flunitrazepam (24%). Variability obscured other changes in modulation of [^{3}H]-muscimol binding by flunitrazepam in saline and cocaine treated rats.

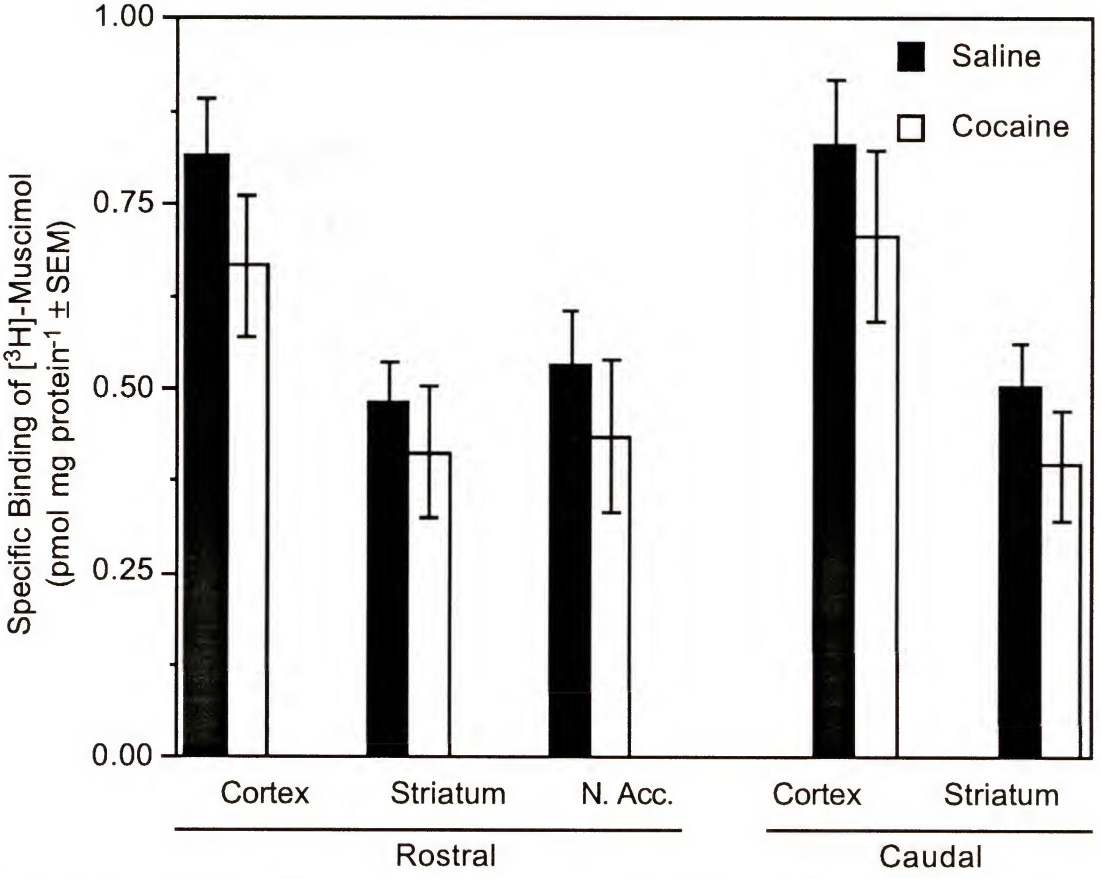

Figure 5-5 Effects of repeated cocaine on specific [^{3}H]-muscimol binding modulated by flunitrazepam. Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 24 hr after the last treatment. There was no significant difference between the two treatment groups. The cortex in both the rostral and caudal slices had significantly increased [^{3}H]-muscimol binding (rostral, $p < 0.01$; caudal, $p < 0.001$). Data are expressed as the mean ± SEM for N = 11.

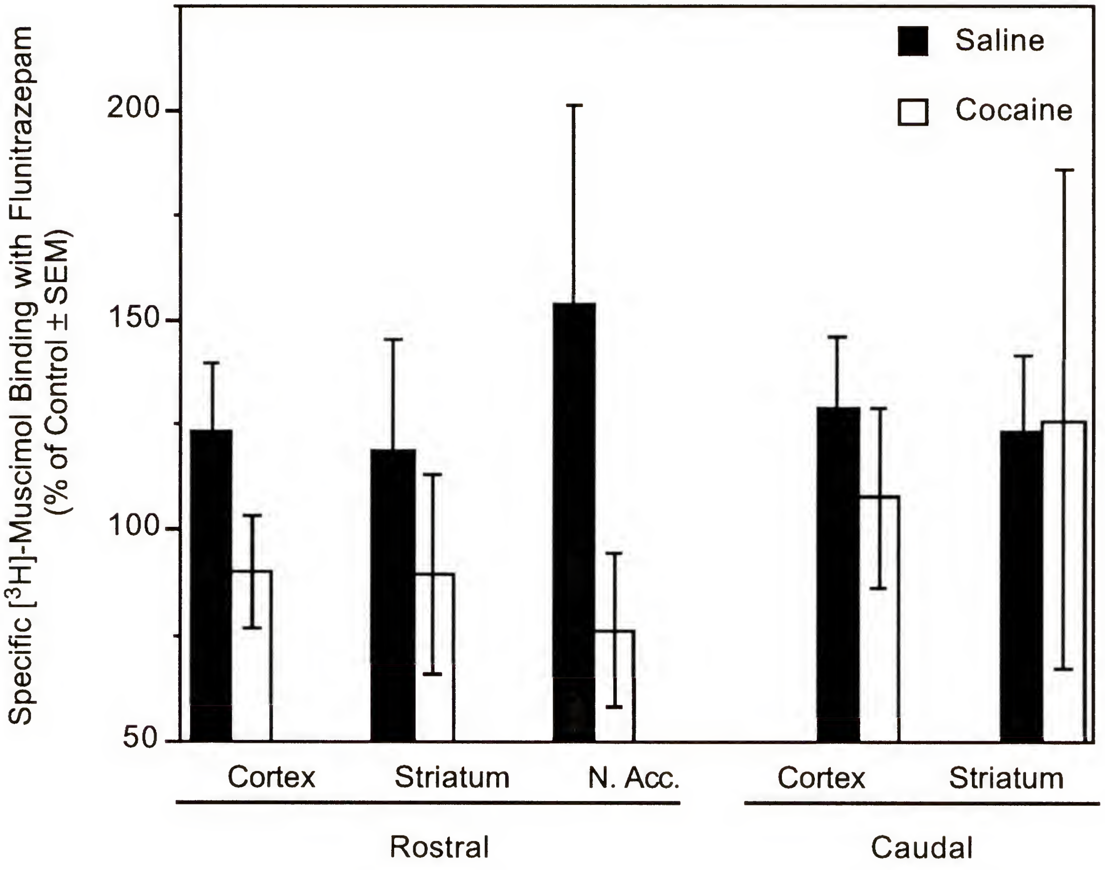

Figure 5-6 The modulation of specific [^{3}H]-muscimol binding with the addition of flunitrazepam. [^{3}H]-Muscimol binding with flunitrazepam was compared to [^{3}H]-muscimol binding alone. Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 24 hr after the last treatment. The percentage of binding was calculated by the amount of [^{3}H]-muscimol binding in the presence of flunitrazepam divided by the amount of [^{3}H]-muscimol binding alone. Data are expressed as the mean percentage ± SEM for N = 10 – 11.

QAR of [^{3}H]-Flunitrazepam Binding

The repeated administration of cocaine resulted in little or no effects on the number of GABA binding sites as measured by [^{3}H]-flunitrazepam binding in any of the regions measured (Figure 5-7). The cortex of both saline and cocaine treated rats had increased [^{3}H]-flunitrazepam binding relative to the STR and NAc for both rostral slices [F(2, 60) = 39.32, $p < 0.0001$] and caudal slices [F(1, 40) = 36.92, $p < 0.0001$].

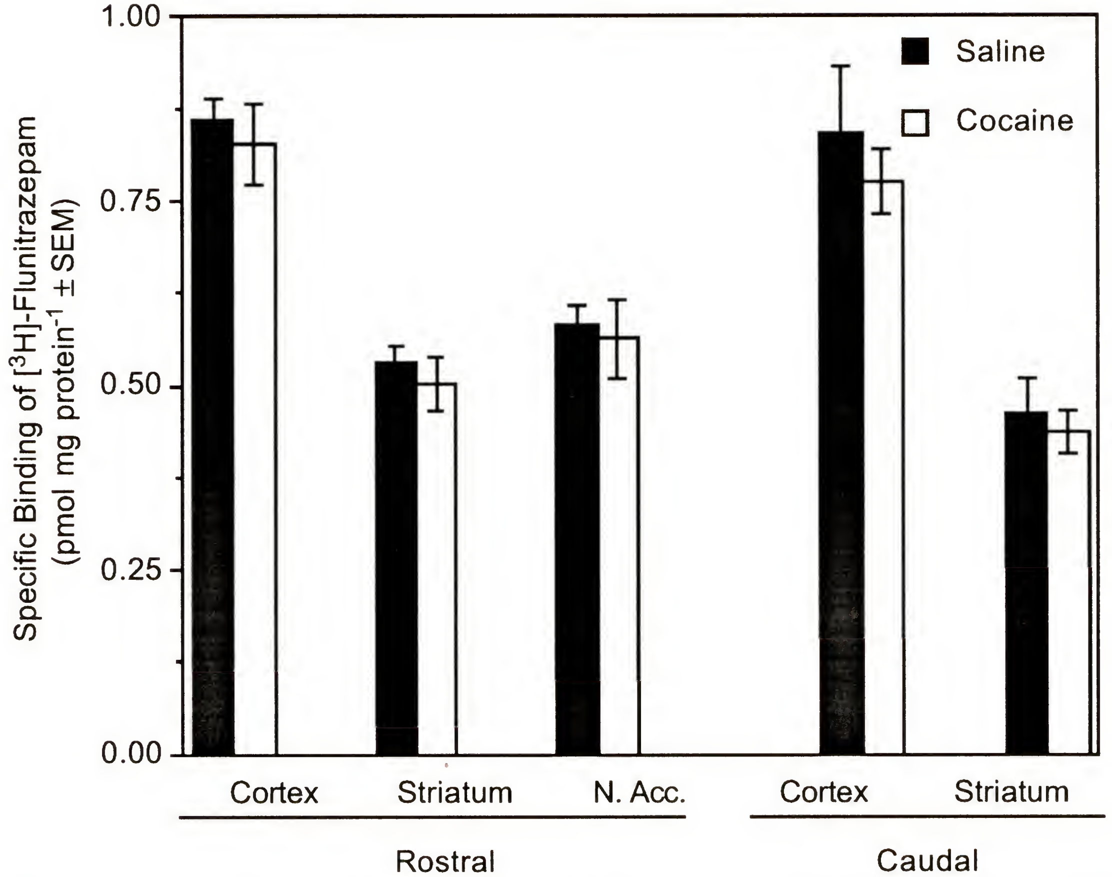

Figure 5-7 Effects of repeated cocaine on specific [^{3}H]-flunitrazepam binding. Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 24 hr after the last treatment. There was no significant difference between the two treatment groups. The cortex in both the rostral and caudal slices had significantly increased [^{3}H]-flunitrazepam binding (rostral, $p < 0.0001$; caudal, $p < 0.0001$). Data are expressed as the mean ± SEM for N = 11.

The repeated administration of cocaine also had little to no effects on [^{3}H]-flunitrazepam binding modulated by GABA in any of the regions measured (Figure 5-8). Again, the cortex had increased [^{3}H]-flunitrazepam binding relative to the STR and NAc for rostral slices [$F(2, 60) = 78.37$, $p < 0.0001$] and for caudal slices [$F(1, 36) = 252.17$, $p < 0.0001$]. The modulation of [^{3}H]-flunitrazepam binding with GABA was next examined (Figure 5-9). GABA enhanced [^{3}H]-flunitrazepam binding between 61% and 99%. There was a significant interaction between enhanced GABA-modulated [^{3}H]-flunitrazepam binding and brain regions for both rostral slices [$F(2, 120) = 3.84$, $p < 0.05$] and caudal slices [$F(1, 76) = 12.85$, $p < 0.001$]. However, there were no significant differences in enhancement between treatment groups.

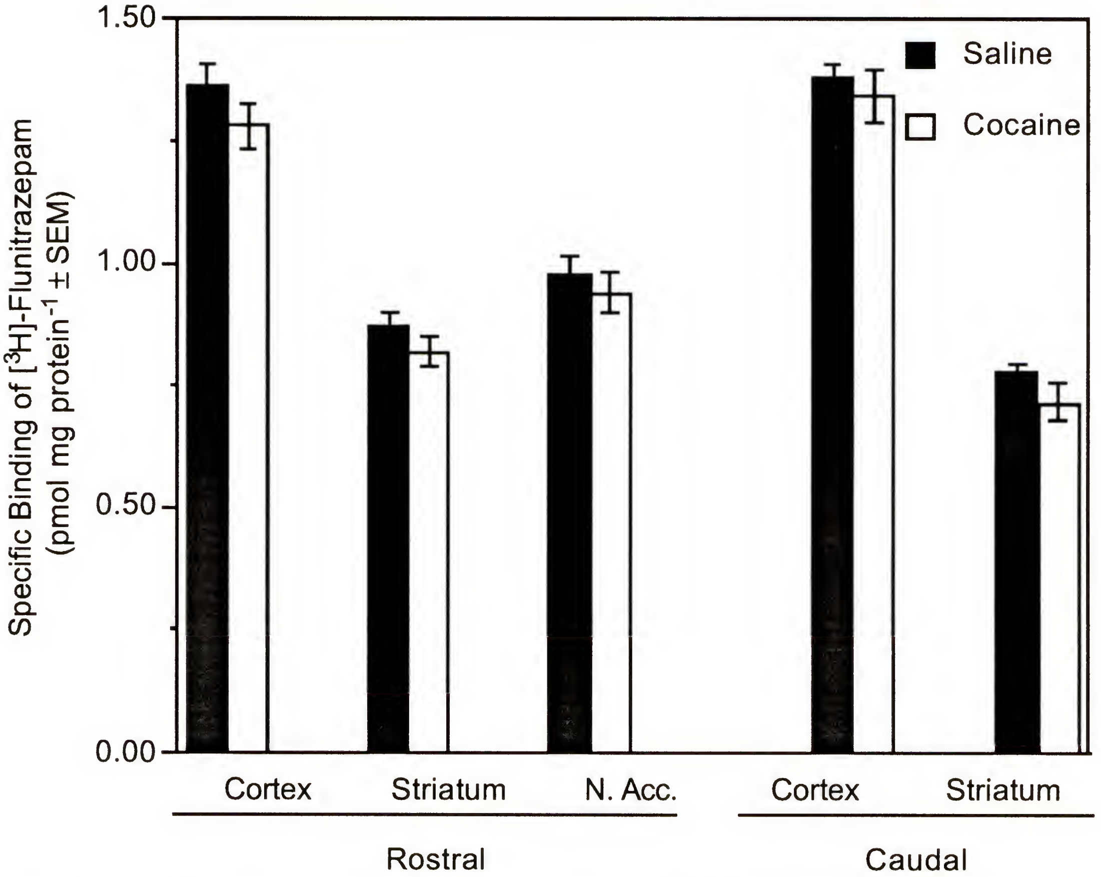

Figure 5-8 Effects of repeated cocaine on specific [^{3}H]-flunitrazepam binding modulated by GABA. Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 24 hr after the last treatment. There was no significant difference between the two treatment groups. The cortex in both the rostral and caudal slices had significantly increased [^{3}H]-muscimol binding (rostral, $p < 0.0001$; caudal, $p < 0.0001$). Data are expressed as the mean percentage change ± SEM for rostral (N = 11) and caudal (N = 10).

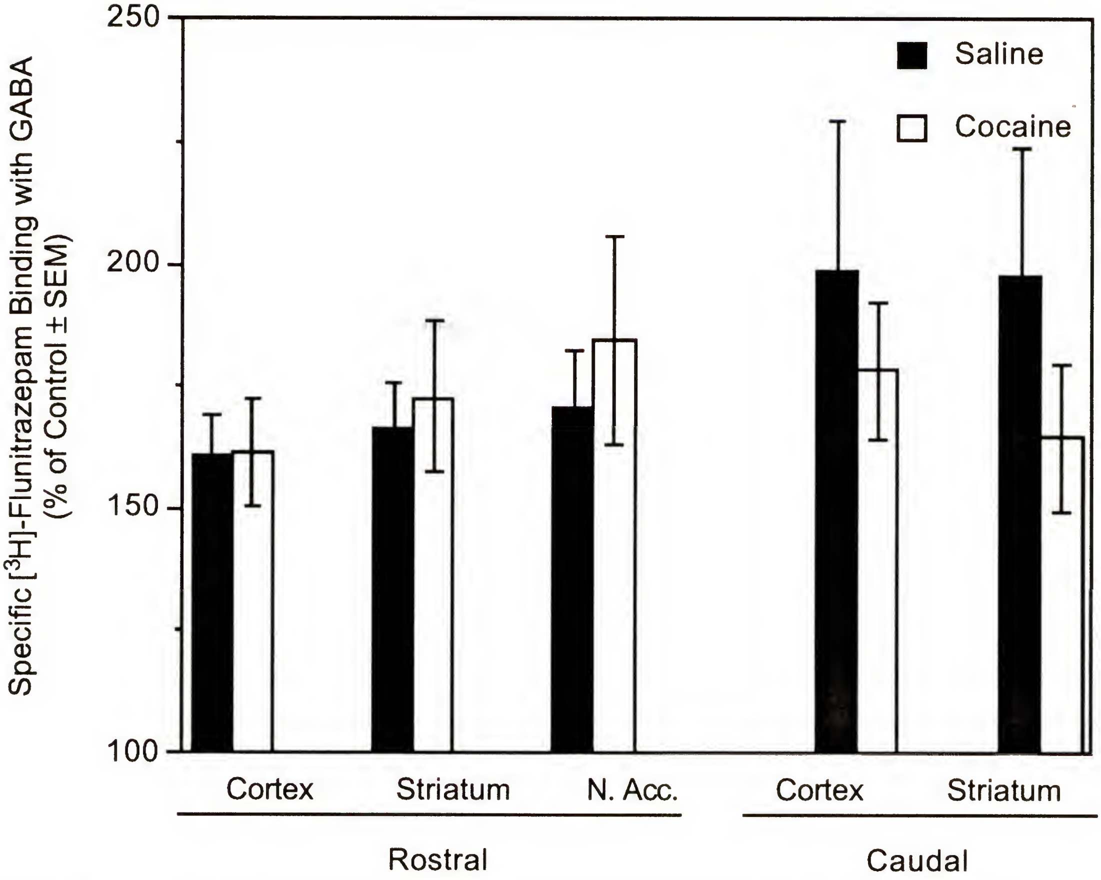

Figure 5-9 The modulation of [^{3}H]-flunitrazepam binding with the addition of GABA. GABA enhanced [^{3}H]-flunitrazepam binding in all regions measured. [^{3}H]-Flunitrazepam binding with GABA was compared to [^{3}H]-flunitrazepam binding alone. The GABA enhancement was similar between saline and cocaine treated rats. There was a significant interaction between enhanced GABA-modulated [^{3}H]-flunitrazepam binding and brain regions for rostral slices [F(2, 120) = 3.84, $p < 0.05$] and for caudal slices [F(1, 76) = 12.85, $p < 0.001$]. The percentage of binding was calculated by the amount of [^{3}H]-flunitrazepam binding in the presence of GABA divided by the amount of [^{3}H]-flunitrazepam binding alone. Data are expressed as the mean percentage ± SEM for rostral (N = 11) and caudal (N = 10).

Discussion

Previous reports characterizing changes in GABA$_A$ receptor number after repeated cocaine have been equivocal (Goeders, 1991; Pecins-Thompson and Peris, 1993). A major difference was the type of ligand used to identify the GABA$_A$ receptor. Goeders used [^{3}H]-Ro 15-1788, a BZD, to label the GABA$_A$ receptors. Pecins-Thompson and Peris used [^{35}S]-TBPS, which binds to the convulsive site on the receptor. Consequently, the reported changes do not measure changes to the neurotransmitter site, but rather the non-competitive binding sites. In addition, the previous studies did not address the ability of binding to the $GABA_A$ receptor to be allosterically modulated after repeated cocaine. Thus, it was important to determine two features about the $GABA_A$ receptor after repeated cocaine; (1) changes in muscimol binding, and (2) whether binding to the receptor could be functionally modulated by an allosteric ligand.

When rat behavior was measured, Group C15 rats had significantly higher behavior on Day 14 compared to Day 1. The variability of behaviors of Group C15 rats on Day 1 and 14 were within the range of behaviors seen in the previous study. However, only eight of 11 Group C15 rats showed an increase in behavioral score on Day 14. It is possible that in this case the injection of cocaine was not administered properly because the technician was nervous. The three Group C15 rats that did not show an increase in behavioral score may have contributed to the variability in the binding assays, but since I administered the cocaine dose on the other days preceding behavior I have confidence that they received repeated cocaine at other time points.

Binding to the $GABA_A$ receptors was determined with two different ligands: muscimol and flunitrazepam. Muscimol binds to the GABA recognition site on the $GABA_A$ receptor and is representative of changes in $GABA_A$ receptor number (Sieghart, 1992). After 14 days of repeated cocaine, there was no change in [^{3}H]-muscimol binding in the regions studied (CTX, STR and NAc). Neither were there changes in [^{3}H]-flunitrazepam binding in the same studied regions. These results indicate that the actual number of $GABA_A$ receptors were not changed after withdrawal from repeated cocaine, and that the previous changes in $GABA_A$ receptor number may represent alternative changes, e.g. receptor decoupling, changes in endogenous benzodiazepines, or changes in receptor subunits.

The ability of allosteric ligands to modulate the $GABA_A$ receptor is an important measure of the function of the receptor. Chronic BZD treatment results in uncoupling of the BZD binding site from the GABA site of the $GABA_A$ receptor (Gallager *et al.*, 1984; Mele *et al.*, 1984; Tietz *et al.*, 1989). In the present experiments, however, repeated cocaine did not change functional coupling when measured either at the BZD site (Figure 5-8) or the GABA site (Figure 5-6). Consequently, the previously observed increase in BZD binding does not appear to be caused by uncoupling of the $GABA_A$ receptor after repeated cocaine. The lack of uncoupling and no difference in $[^3H]$-flunitrazepam binding after one day withdrawal could also indicate that the $GABA_A$ receptor has reverted back to the non-sensitized form. Goeders observed the increase in BZD binding, 20 min after withdrawal, but not two and 14 days after withdrawal (Goeders, 1991). This result also emphasizes the importance of evaluating measures of sensitization at several points after withdrawal, since cocaine sensitization is known to be persistent. In addition, it is also possible that the binding curve or affinity for $[^3H]$-flunitrazepam was shifted, which was not measured in these experiments. The ability of chronic drug treatment to shift the affinity of $[^3H]$-flunitrazepam (GABA shift) has been demonstrated with other $GABA_A$ receptor agonists such as muscimol and flunitrazepam (Klein *et al.*, 1995). Thus, although repeated cocaine did not affect functional binding by uncoupling the GABA site from the BZD site, the affinity of either the GABA or BZD site could still change functional binding.

$[^3H]$-Muscimol binding has been reported to occur at two affinity sites with K_Ds of 2.2 nM and 60 nM (Beumont, *et al.*, 1978). In this study, QAR binding was performed with 30 nM $[^3H]$-muscimol. Hence, the high affinity site should have been completely labeled. However, specific muscimol binding data were low when compared to previous reports (Beumont *et al.*, 1978; Palacios *et al.*, 1981). The low specificity seemed to affect the variability in the modulation of $[^3H]$-muscimol binding by flunitrazepam such that significant enhancement did not occur. $[^3H]$-Muscimol binding to the GABA site may have been affected by endogenous GABA. It is well known that completely removing endogenous GABA from brain tissue is difficult. $[^3H]$-Muscimol binding may have been improved by more thorough washing, but this may also compromise the integrity of the brain tissue. If endogenous GABA interfered with [^{3}H]-muscimol binding, then it is most likely that the low affinity [^{3}H]-muscimol binding site was only partially occupied in these experiments. Consequently, the presence of endogenous GABA may have obscured differences between [^{3}H]-muscimol binding after saline and cocaine treatments. Both the specificity of binding of [^{3}H]-flunitrazepam and [^{3}H]-flunitrazepam modulated binding are consistent with previous reports (Ruano *et al.*, 1992; Slaney *et al.*, 1995). Thus, the results of both [^{3}H]-flunitrazepam binding and [^{3}H]-flunitrazepam modulated binding provide better support for no difference in binding and receptor coupling after repeated cocaine.

The experiments here still do not resolve the differences obtained in the two laboratories. Originally, I hypothesized that the differences could be accounted for by uncoupling of the GABA site from the non-competitive BZD site. The results from [^{3}H]-muscimol binding are not clear, where there were trends towards a difference between the two treatment groups with great variability (Figure 5-6). It may be necessary to both improve [^{3}H]-muscimol QAR and also measure changes in binding affinity to determine if there are real differences between the two treatment groups. The results from [^{3}H]-flunitrazepam binding and modulated [^{3}H]-flunitrazepam strongly suggest that uncoupling does not occur after one day withdrawal from repeated cocaine. It would be important to determine if the GABA$_A$ receptor is decoupled at earlier periods after withdrawal because the reason for the increase in BZD binding after 20 min withdrawal from repeated cocaine is unknown. If the increase in BZD binding was not caused by uncoupling of the GABA$_A$ receptor, then changes in endogenous benzodiazepine or GABA$_A$ receptor subunits could account for the increase in BZD binding. The release of the endogenous benzodiazepine, endozepine, is regulated by GABA through the GABA$_B$ receptors (Patte *et al.*, 1999). It is possible that repeated cocaine is affecting the production and release of endozepine. Another possibility is that the GABA$_A$ receptor subunits are changed to increase the amount of BZD binding sites after repeated cocaine. It would be informative to determine the changes in $GABA_A$ receptor subunit composition and number after cocaine sensitization after withdrawal at multiple time points. In an early report, $GABA_A$ receptor subunit mRNAs for α_1 and others have been reported to not change in the STR after repeated cocaine, but subunit expression was not measured (Olinger *et al.*, 1999).

In summary, repeated cocaine did not alter the number of $GABA_A$ receptors as measured by $[^3H]$-muscimol binding or $[^3H]$-flunitrazepam binding. The lack of change in $[^3H]$-muscimol binding was surprising because of previously reported changes in $GABA_A$ receptor number by BZD and convulsant binding. The GABA binding site was not decoupled from the allosteric binding site, but it is possible that repeated cocaine affects the affinity of the receptor, which was not measured, more than number at these allosteric sites. Thus, repeated cocaine does not appear to change coupling of the $GABA_A$ receptor after one day withdrawal.

CHAPTER 6
FUNCTIONAL MODULATION OF STIMULATED $GABA_A$ RECEPTOR $^{36}CL^-$ UPTAKE AFTER COCAINE SENSITIZATION

Introduction

The $GABA_A$ receptor possesses multiple ligand binding sites that can affect the functional properties of the receptor (Sieghart, 1992). As discussed in the previous chapter, functional binding can be used to describe modulation of $GABA_A$ receptor function by allosteric binding sites. In the previous chapter, I examined coupling of the GABA and BZD sites in terms of binding and found that it was unchanged after cocaine sensitization. Although the binding sites remain coupled after cocaine sensitization, allosteric regulation of $^{36}Cl^-$ uptake by the $GABA_A$ receptor may be altered after cocaine sensitization.

In some animal models of tolerance to either ethanol or BZD, the BZD allosteric site was shown to be decoupled from the $GABA_A$ receptor. Decoupling was demonstrated by decreased $GABA_A$ receptor $^{36}Cl^-$ uptake in the presence of a BZD compound (Yu *et al.*, 1988; Buck and Harris, 1990b; Allan *et al.*, 1992; Li *et al.*, 1993). In addition, there was no reported difference in BZD B_{max} with muscimol or GABA modulation (Buck and Harris, 1990b; Allan *et al.*, 1992). In these cases, decoupling of the BZD allosteric site from the $GABA_A$ receptor is a reasonable adaptation to decrease enhancement caused by either ethanol or BZD. After repeated cocaine, there are also decreases in striatal GABA release (Chapter 3) and in striatal $GABA_A$ receptor function (Peris, 1996). If the BZD site is decoupled from the $GABA_A$ receptor after repeated cocaine, this would further strengthen the role for decreased striatal GABAergic neurotransmission in cocaine sensitization and possibly explain the discrepancies in binding to the $GABA_A$ receptor after cocaine sensitization.

In the previous chapters, the repeated treatment with cocaine (15 mg kg^{-1}) led to the higher behavioral scores on Day 14 compared to Day 1 in 80% of the rats. Although there was a significant increase in behavioral score when Day 8 or 14 was compared to Day 1, I believe that by increasing the dose of cocaine to 20 mg kg^{-1} that this dose would increase the number of animals developing sensitization when Day 14 was compared to Day 1. In addition since the scores remained on the lower end of the rating scale, cocaine sensitized behavior could still be increased. Thus, the dose of cocaine was changed to increase the percentage of rats being sensitized on Day 14 and to increase the range on the rating scale.

Previously, the effects of *in vitro* cocaine has been examined on GABA-stimulated $^{36}Cl^-$ uptake in microsacs, but without the inclusion of protease inhibitors (Peris, 1996). The purpose of that study was to show that the remaining cocaine after withdrawal, which should be little to none, was not causing the decrease in striatal GABA-stimulated $^{36}Cl^-$ uptake after cocaine withdrawal. Originally, they observed that there was no change in $GABA_A$ receptor function with the addition of *in vitro* cocaine. However from preliminary data obtained with the inclusion of protease inhibitors in the assay buffer, an effect of *in vitro* cocaine has been observed. Hence, I was interested in determining if *in vitro* cocaine had a greater effect on striatal GABA-stimulated $^{36}Cl^-$ uptake after repeated cocaine compared to control.

In this study, stimulated $GABA_A$ receptor function and modulation of stimulated $GABA_A$ receptor function was measured after repeated cocaine. Since the previous reports by Goeders (1991), Pecins-Thompson and Peris (1993), and Peris (1996) did not measure the status of modulation of stimulated $GABA_A$ receptor function after repeated cocaine, it would be informative to evaluate the modulation of uptake. Although the binding sites were not decoupled after cocaine sensitization (Chapter 5), the allosteric ligand site may still be shown to be decoupled through changes in modulation of stimulated $GABA_A$ receptor function. The results obtained showed that exogenous

BZD increases in striatal $GABA_A$ receptor function were not changed after cocaine sensitization. In addition, *in vitro* cocaine decreased stimulated $GABA_A$ receptor $^{36}Cl^-$ uptake in both saline and cocaine treated rats to a similar degree.

Methods

Animals and Treatment

Male Sprague-Dawley rats were used and housed as described in Chapter 2. For cocaine sensitization, the rats were treated as follows; after three daily saline injections (1.0 ml kg^{-1} i.p.) in the home cages, the drug treatment regimen started. Rats received either 1.0 ml kg^{-1} saline i.p. (Group S, N = 11) or 20 mg kg^{-1} cocaine i.p. (Group C20, N = 10). In this study, two separate groups of treated rats were prepared at separate times. In the first set of treated rats, one of the cocaine treated rats was sick and subsequently euthanized. Rats were killed 24 hr after their last injection to obtain striatal and cortical tissue.

Behavioral Rating

Behavior was measured as described in Chapter 2. Behavior was rated for 10 sec every 2.5 min for 60 min. Rats were determined to be sensitized if there was a significant increase in behavioral scores after Day 1.

GABA-Stimulated $^{36}Cl^-$ Uptake in Brain Microsacs

Brain microsacs from the striatum and cortex were prepared as described in Chapter 2. Briefly, brains were homogenized using a Potter-Elvehjem tissue grinder in ice-cold Ca^{2+} buffer containing protease inhibitors. Homogenates were centrifuged at 900 x *g* for 15 min using a Sorvall SA600 rotor; supernatants were discarded; pellets were resuspended and centrifuged again. The resulting pellets (microsacs) were suspended in Ca^{2+} buffer with protease inhibitors to yield 800 – 1600 μg of protein per ml.

Next, microsacs were pre-incubated for 5 min at 34°C. The subsequent 3 sec incubation (uptake period) contained both $^{36}Cl^-$ and treatment drugs. Assays with drug pretreatment included the drug in the 5 min pre-incubation period as well as during the uptake period. Uptake was terminated by addition of Ca^{2+} buffer with 100 μM picrotoxin and followed by vacuum filtration onto a glass fiber filter. Two additional washes of Ca^{2+} buffer with picrotoxin were applied onto the filter. The amount of $^{36}Cl^-$ retained on the filter was determined by liquid scintillation. Stimulated $^{36}Cl^-$ uptake was defined as the amount of $^{36}Cl^-$ taken up while agonist was present minus the amount of $^{36}Cl^-$ taken up when agonist was not present. GABA (20 μM) was used as the $GABA_A$ receptor agonist. Cocaine (10 μM) was added during both pre-incubation and uptake periods. Clonazepam is a BZD that increases $GABA_A$ receptor $^{36}Cl^-$ uptake in the presence of GABA. Clonazepam (20 μM) was included only during the uptake period (3 sec) in the assays.

Data Analysis

The data are expressed as the mean ± SEM; N = number of subjects. For statistical analysis, Wilcoxon signed-rank test was used to analyze behavioral data. Mann-Whitney U was used to determine within group differences. ANOVA was used on unnormalized $^{36}Cl^-$ uptake data. Bonferroni-Dunn was used for *post-hoc* analysis when appropriate. A p value less than 0.05 was considered to be significant.

Results

Behavior

Rats were treated with either repeated saline or cocaine injections. Rat behavior was rated on Day 1, 7 and 14 (Figure 6-1). Group S treatment did not result in a significant change in behavior across days. However, Group C20 treatment resulted in a significant increase in behavior when Day 14 was compared to Day 1 ($p < 0.01$). In this

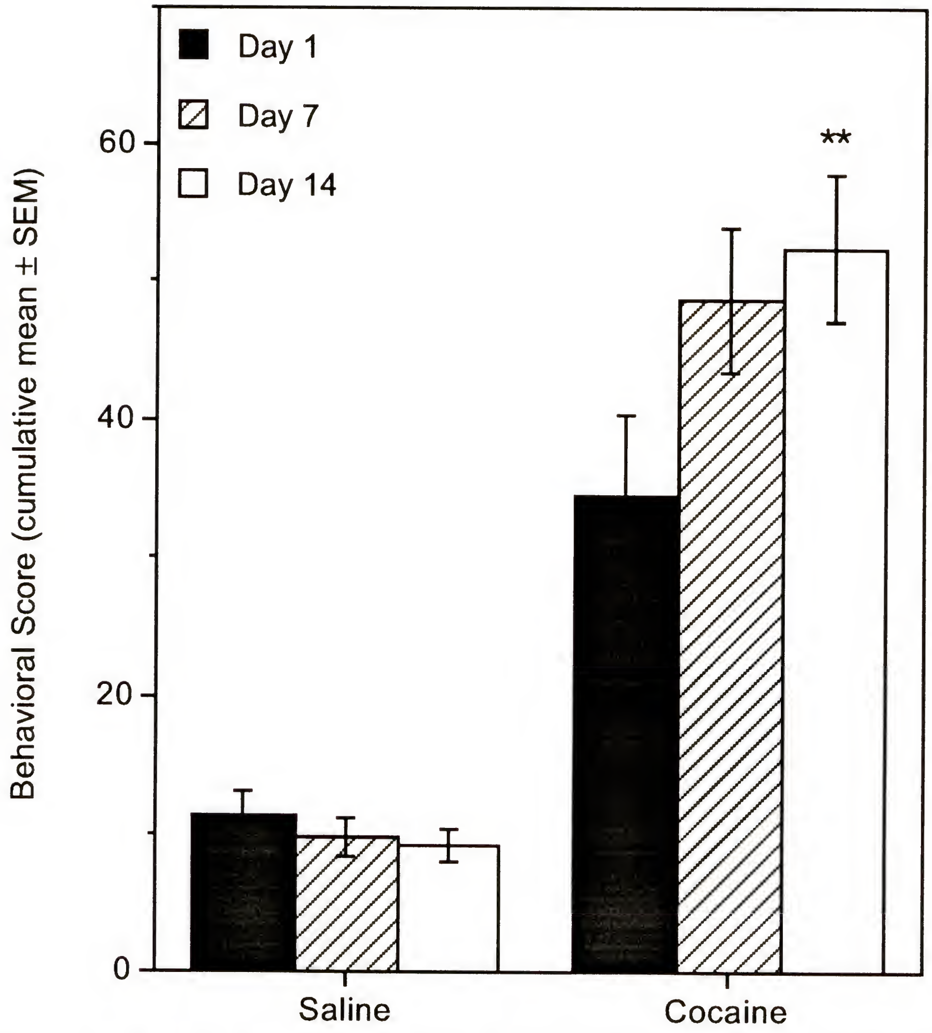

Figure 6-1 Cumulative behavioral scores of saline (0.1 ml kg^{-1}) and cocaine (20 mg kg^{-1}) treated rats on Day 1, 7 and 14. Behavior was rated for 10 sec every 2.5 min for 60 min. Daily cocaine treatment led to a significantly greater behavior on Day 14 compared to Day 1 ($p < 0.01$). The cumulative behavioral scores within treatment groups were compared across days using the Wilcoxon signed-rank test. Data are expressed as the cumulative mean ± SEM for N = 10 – 11 per group. ** $p < 0.01$ vs. Day 1 Cocaine.

study, two separate groups of treated rats were prepared. In the first group of cocaine treated rats, only four of the five surviving cocaine treated rats showed a higher behavioral scores when Day 14 is compared to Day 1 (data not shown). In the second group of cocaine treated rats, all rats (5) showed a higher behavioral score when Day 14 is compared to Day 1 (data not shown). The behavioral scores between cocaine treated rats on Day 1 and Day 14 were not significantly different and were combined for ease of comparison. However, there was a significant difference between the two groups of cocaine treated rats on Day 7 ($p < 0.05$) with the first group receiving significantly higher cumulative behavioral scores than the second group of cocaine treated rats (data not shown).

Striatal and Cortical Microsac $^{36}Cl^-$ Uptake after Cocaine Sensitization

After treatment with either repeated saline or cocaine, brain microsacs were prepared from the striatum and cerebral cortex from these rats. When striatal GABA-stimulated $^{36}Cl^-$ uptake was measured, there were no differences between repeated saline and cocaine treated rats shown in Figure 6-2 or Figure 6-3 (normalized). Next functional modulation of striatal $GABA_A$ receptor function was examined with clonazepam and/or cocaine. ANOVA revealed that clonazepam significantly enhanced striatal $^{36}Cl^-$ uptake in both the saline and cocaine treated rats [$F(1, 68) = 72.64, p < 0.0001$], but there were no differences between repeated saline and cocaine groups. ANOVA also revealed that *in vitro* cocaine significantly decreased striatal $^{36}Cl^-$ uptake in both saline and cocaine treated rats [$F(1, 68 = 4.68, p < 0.05$], but again there were no differences between the treatment groups.

When cortical GABA-stimulated uptake was measured, there were no differences between repeated saline and cocaine treated rats shown in Figure 6-5 or Figure6-6 (normalized). Again clonazepam modulation of $GABA_A$ receptor function significantly increased cortical $^{36}Cl^-$ uptake [$F(1,76) = 63.53, p < 0.0001$], but there were no differences between saline and cocaine treated rats. In contrast to the striatum, *in vitro* cocaine did not have a significant effect on cortical $GABA_A$ receptor function. When the effects of both clonazepam and *in vitro* cocaine were examined, clonazepam affected cortical GABA-stimulated $^{36}Cl^-$ uptake similar to when no cocaine was present.

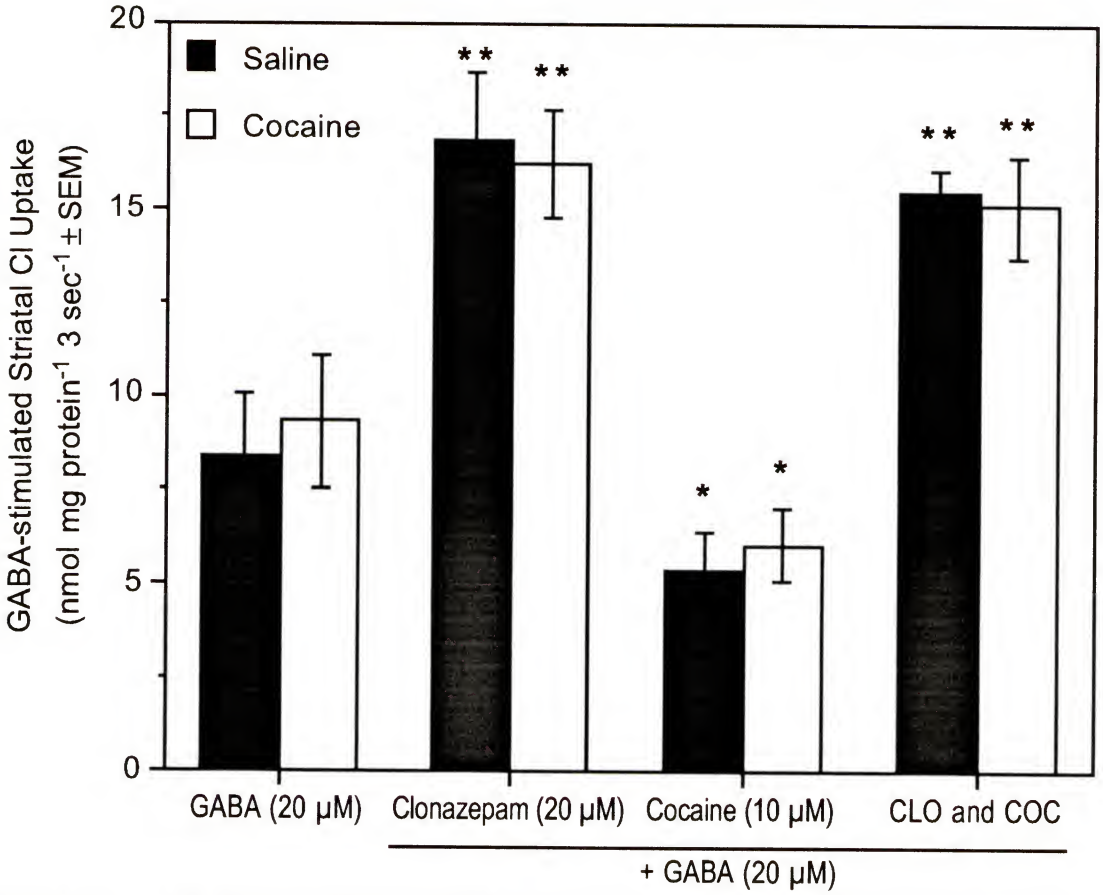

Figure 6-2 Changes in striatal GABA-stimulated $^{36}Cl^-$ uptake after cocaine sensitization with the addition of *in vitro* clonazepam or *in vitro* cocaine. *In vitro* clonazepam significantly enhanced [F(1, 68) = 72.64 , $p < 0.0001$] and *in vitro* cocaine significantly decreased striatal $^{36}Cl^-$ uptake [F(1, 68 = 4.68, $p < 0.05$]. There was no difference in $^{36}Cl^-$ uptake between repeated *in vivo* saline and cocaine treatment. Data are expressed as the mean ± SEM for N = 10 – 11. * $p < 0.05$ compared to GABA (20 μM) alone; ** $p < 0.0001$ compared to GABA (20 μM) alone.

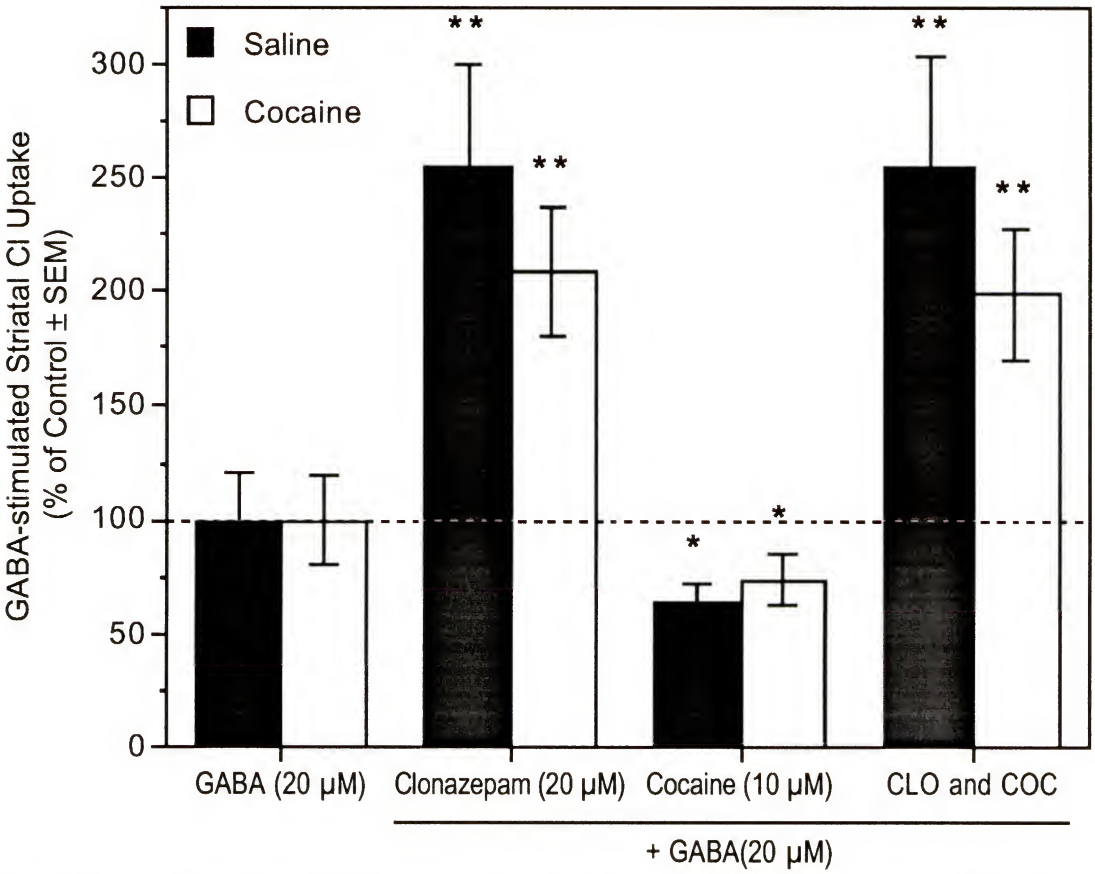

Figure 6-3 The effects of repeated cocaine on striatal GABA-stimulated $^{36}Cl^-$ uptake with modulation of response by *in vitro* clonazepam and *in vitro* cocaine. These data are from Figure 6-2 and re-presented as a percentage of control. *In vitro* clonazepam significantly enhanced [F(1, 68) = 72.64 , $p < 0.0001$] and *in vitro* cocaine significantly decreased striatal $^{36}Cl^-$ uptake [F(1, 68 = 4.68, $p < 0.05$]. There was no difference in $^{36}Cl^-$ uptake between repeated *in vivo* saline and cocaine treatment. Data are expressed as the mean percentage of experimental control ± SEM for N = 10 – 11. * $p < 0.05$ compared to GABA (20 µM) alone; ** $p < 0.0001$ compared to GABA (20 µM) alone.

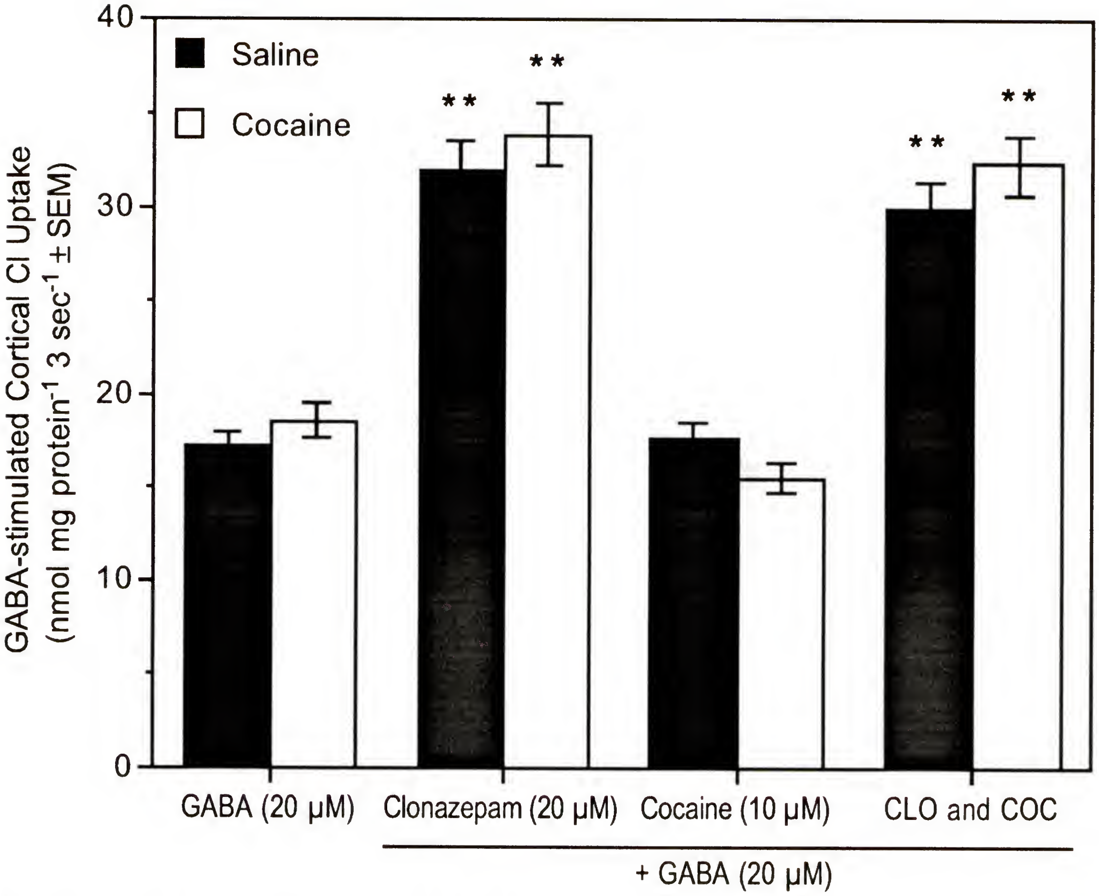

Figure 6-4 Changes in cortical GABA-stimulated $^{36}Cl^-$ uptake after cocaine sensitization with the addition of *in vitro* clonazepam or *in vitro* cocaine. *In vitro* clonazepam significantly enhanced cortical $^{36}Cl^-$ uptake [$F(1,76) = 63.53, p < 0.0001$] in both treatment groups. There was no difference in $^{36}Cl^-$ uptake between repeated *in vivo* saline and cocaine treatment. Data are expressed as the mean ± SEM for N = 10 – 11. ** $p < 0.0001$ compared to GABA (20 µM) alone.

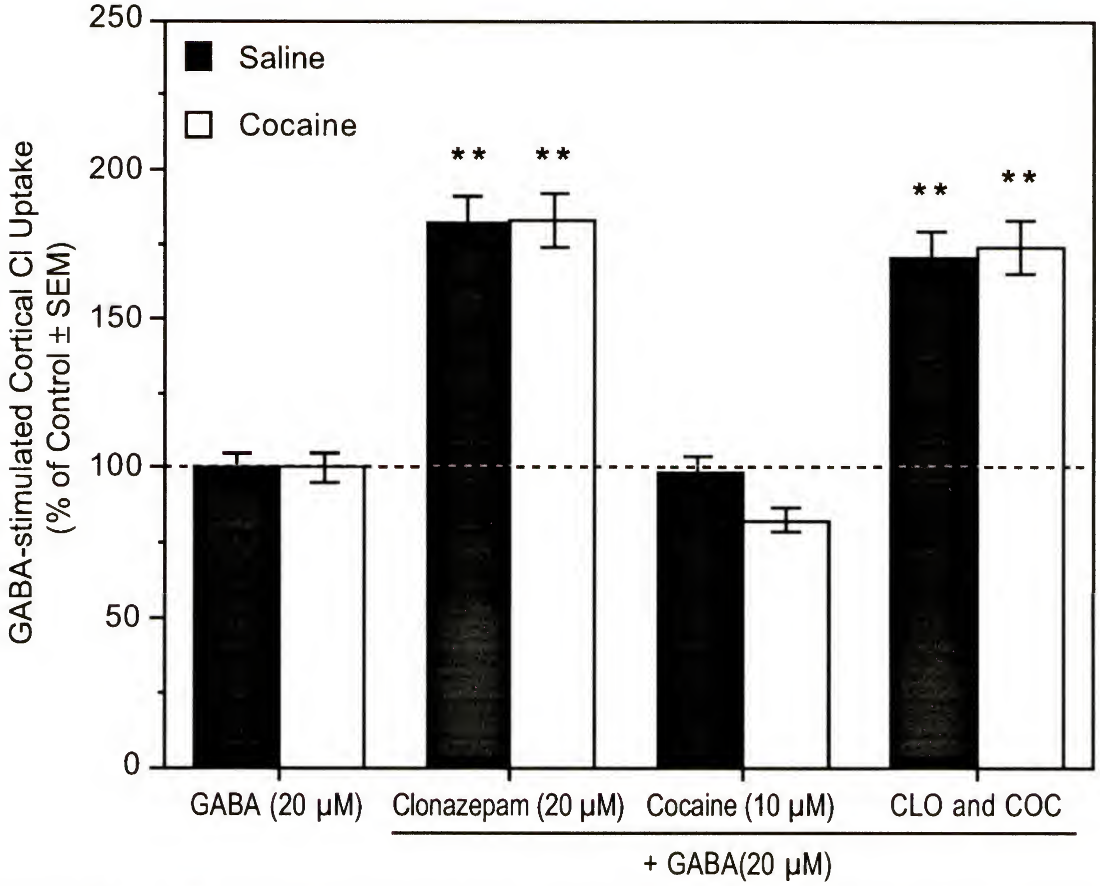

Figure 6-5 Changes in cortical GABA-stimulated $^{36}Cl^-$ uptake after cocaine sensitization with the addition of *in vitro* clonazepam or *in vitro* cocaine. These data are from Figure 6-4 and re-presented as a percentage of control. *In vitro* clonazepam significantly enhanced cortical $^{36}Cl^-$ uptake. There was no difference in $^{36}Cl^-$ uptake between repeated *in vivo* saline and cocaine treatment. Data are expressed as the mean percentage of experimental control ± SEM for N = 10 – 11. ** $p < 0.0001$ compared to GABA (20 μM) alone.

Discussion

Previously after repeated cocaine, striatal $GABA_A$ receptor binding and function were decreased (Pecins-Thompson and Peris, 1993; Peris, 1996). These reports did not describe the response of the $GABA_A$ receptor to allosteric modulators of function, either binding or uptake. Previously in Chapter 5, I described the effects of cocaine sensitization on binding of allosteric ligands to the $GABA_A$ receptors. I found that the GABA binding site and the allosteric BZD site remained coupled after cocaine sensitization. In the present study, I sought to determine if allosterically modulated striatal $GABA_A$ receptor $^{36}Cl^-$ uptake was affected by cocaine sensitization.

The ability of allosteric ligands to modulate the $GABA_A$ receptor is an important measure of the receptor's function. BZD agonists enhanced the uptake of $^{36}Cl^-$ by the $GABA_A$ receptor (Buck and Harris, 1990a; Allan *et al.*, 1992; Mihic *et al.*, 1992; Francis *et al.*, 1994). In the present study, clonazepam modulated $GABA_A$ receptor $^{36}Cl^-$ uptake was measured in both striatal and cortical microsacs. Clonazepam significantly enhanced cortical GABA-stimulated $^{36}Cl^-$ uptake similar to the previously reported E_{max} at similar concentrations of both GABA and clonazepam (Li *et al.*, 1993; Francis *et al.*, 1994). In Figure 6-3, it appears as though striatal GABA-stimulated $^{36}Cl^-$ uptake with either clonazepam alone or clonazepam and *in vitro* cocaine was decreased in cocaine treated rats, but this may be attributed to one of the saline rats having greatly enhanced stimulated $^{36}Cl^-$ uptake, which also contributed to the variability. Lastly in Figure 6-6, it appears as though *in vitro* cocaine after repeated cocaine shows a trend towards decreased cortical GABA-stimulated $^{36}Cl^-$ uptake. This trend may be due to the starting 10% difference in GABA-stimulated $^{36}Cl^-$ uptake alone and then an additional 10% difference in the effect of *in vitro* cocaine on GABA-stimulated $^{36}Cl^-$ uptake between repeated saline and cocaine treated rats. However from the normalized data, it appears as if in cocaine-treated rats there is a trend towards lower GABA-stimulated uptake than the controls. It is possible that repeated cocaine is affecting other neurotransmitters than dopamine in the CTX.

Chronic BZD treatment results in a decoupling of the GABA binding site from the BZD site as measured by GABA-stimulated $^{36}Cl^-$ uptake (Yu *et al.*, 1988; Allan *et al.*, 1992; Li *et al.*, 1993). Chronic ethanol also leads to decoupling of the $GABA_A$ receptor's BZD binding site from the GABA binding site (Buck and Harris, 1990b). In the present study, the BZD ligand, clonazepam, retained the ability to modulate both striatal and cortical $GABA_A$ receptor $^{36}Cl^-$ uptake after repeated cocaine. Previous reports of

$GABA_A$ receptor $^{36}Cl^-$ uptake decoupling have all shown significant decreases in BZD enhancement of $GABA_A$ receptor $^{36}Cl^-$ uptake at the E_{max} of the BZD used and a low $GABA_A$ receptor agonist concentration (Yu *et al.*, 1988; Buck and Harris, 1990b; Li *et al.*, 1993). Thus, it is unlikely that different concentrations of GABA or clonazepam would have affected the results.

The time of withdrawal after the last drug treatment may be critical in the demonstration of receptor decoupling. In these previous reports of $GABA_A$ receptor $^{36}Cl^-$ uptake decoupling, the receptor was decoupled from the BZD site immediately (Li *et al.*, 1993) and 6 hr (Buck and Harris, 1990b) after withdrawal, but not 24 hr (Buck and Harris, 1990b) and 48 hr (Li *et al.*, 1993) after. In the present study, the $GABA_A$ receptor $^{36}Cl^-$ uptake was measured 24 hr after the last treatment. It is possible that the receptor may be initially decoupled, then re-couples sometime during this withdrawal period. The possibility that the receptor re-couples the GABA site and BZD site after withdrawal suggests that the allosteric BZD site plays a less significant role in the maintenance of cocaine sensitization because sensitization persists for a longer period than BZD tolerance. It is tempting to speculate that the change from increased BZD binding 20 min after withdrawal (Goeders, 1991) to a lack of difference in BZD binding 24 hr (Chapter 5), two days and 14 days after withdrawal (Goeders, 1991) results from the $GABA_A$ receptor returning to the drug naive state. Thus, the contribution of the allosteric BZD site to the maintenance of cocaine sensitization may be limited.

When rat behavior was measured, Group C20 rats had significantly higher behavior on Day 14 compared to Day 1, but not on Day 7 compared to Day 1 ($p < 0.07$). It is possible that if the study were repeated, behavioral sensitization could occur by Day 7, but this has been shown before with a different dose of cocaine on Day 8 (Peris, 1996). This does suggest that measures of sensitization should be tested early to determine their importance to the initiation or maintenance of sensitization. Group C20 rats had an increased range in the behavioral scores on all days measured, but more importantly also had consistently higher behavioral score on Day 14 compared to Day 1. However, their behavior is not yet maximal with few ratings of the maximal score of 4. At this dose of cocaine (20 mg kg^{-1}), obtaining sensitized rats may be more consistent and possibly using yet a higher dose would yield increased sensitization.

Previously, repeated cocaine treatment decreased striatal $GABA_A$ receptor $^{36}Cl^-$ uptake (Peris, 1996; Resnick *et al.*, 1999). In this study, there was no difference in striatal GABA-stimulated $^{36}Cl^-$ uptake between saline and cocaine treated rats. It is unclear as to why there was no difference in this study because the rats were subjected to the same treatment schedule that resulted in cocaine sensitization. Although the dose of cocaine used to cause cocaine sensitization was greater, this dose increase should theoretically increase behavior and could cause a greater decrease in $GABA_A$ receptor function. Another possibility is that the amount of GABA-stimulated $^{36}Cl^-$ uptake in this study was low compared to previous results (Peris, 1996). The only difference between the two microsac preparations were the inclusion of protease inhibitors in this study, which should have decreased some of the variability between different preparations. In a future experiment it may be critical to show both behavioral sensitization and decreased striatal $GABA_A$ receptor function after repeated cocaine, before examining modulation of GABA-stimulated $^{36}Cl^-$ uptake.

A major difference between the striatum and the cortex is that the striatum contains high concentrations of dopamine transporters, while the cortex has relatively low to no concentrations (Scheffel *et al.*, 1991; Shearman *et al.*, 1996). This difference between the two brain regions could account for *in vitro* cocaine specifically decreasing striatal, but not cortical GABA-stimulated $^{36}Cl^-$ uptake. The mechanism by which *in vitro* cocaine specifically decreases striatal $GABA_A$ receptor $^{36}Cl^-$ uptake is unknown. A mechanism by which *in vitro* cocaine could affect $GABA_A$ receptor function will be addressed in the next chapter, but some additional mechanisms will be considered here. Previously, it was reported from this laboratory that there was no effect of *in vitro* cocaine on striatal $GABA_A$ receptor function, but in that study the microsac preparation did not include protease inhibitors (Peris, 1996). Hence, proteins critical for the effect of *in vitro* cocaine were being degraded in the previous assays. It would be important to identify these proteins in future experiments. The decrease by *in vitro* cocaine was not likely due to a direct effect on the $GABA_A$ receptor. At the present time, there is no reported cocaine binding site on the $GABA_A$ receptor. It has been reported that GABA current is inhibited by cocaine (Ye *et al.*, 1997; Ye *et al.*, 1999), but this occurs at much higher cocaine concentrations and could be the result of a local anesthetic effect of cocaine. More recently, specific subunits of the nicotinic acetylcholine receptor were shown to be inhibited by cocaine and contain a cocaine binding domain (Francis *et al.*, 1999). It is tempting to speculate that the $GABA_A$ receptor subunits may also contain an analogous binding site, since it also belongs to the ligand gated superfamily. Another possibility is that *in vitro* cocaine indirectly downregulates the $GABA_A$ receptor. In a recent report, the D_5 DA receptor directly modulates $GABA_A$ receptor by down regulating the receptor from the cell surface (Man *et al.*, 1999). It is possible that *in vitro* cocaine acting through the D_5 DA receptors could selectively decrease $GABA_A$ receptors. The relative importance of this and the implications of *in vitro* cocaine effect on $GABA_A$ receptor function will be examined further in Chapter 7.

In summary, cocaine sensitization did not result in a change in allosterically modulated striatal $GABA_A$ receptor $^{36}Cl^-$ uptake. In addition, *in vitro* cocaine decreased stimulated $GABA_A$ receptor $^{36}Cl^-$ uptake in both saline and cocaine treated rats. The mechanism by which *in vitro* cocaine specifically decreases striatal $GABA_A$ receptor $^{36}Cl^-$ uptake is unknown and will be examined in the next chapter.

CHAPTER 7
IN VITRO EFFECTS OF COCAINE, FORSKOLIN, AND cAMP-ANALOGS IN NAIVE RATS

Introduction

Cocaine binds to and blocks norepinephrine, DA and 5-HT transporters, with a K_i of 2.93 μM, 1.51 μM, 0.17 μM respectively (Ritz *et al.*, 1987). The concentration of cocaine in the brain has been calculated to range from 1.9 – 5.8 μM after a typical cocaine dose (Ellenhorn and Barceloux, 1988; Horger *et al.*, 1996). At high concentrations (mM), cocaine acts as a local anesthetic by binding to the Na^+ channel and inhibiting ionic flow (Post *et al.*, 1987; Das, 1993). In the last chapter, *in vitro* cocaine decreased $GABA_A$ receptor $^{36}Cl^-$ uptake in both saline and cocaine treated rats. Currently, it is not believed that cocaine has a direct binding site on the $GABA_A$ receptor. However, the application of cocaine directly to rat hippocampal neurons decreased whole cell GABA current (Ye *et al.*, 1997; Ye *et al.*, 1999), but this may be related to the anesthetic effect of cocaine. In another recent report, cocaine was shown to bind to a region of the nicotinic acetylcholine receptor that has some homology to the $GABA_A$ receptor (Francis *et al.*, 1999). However, the mechanism by which *in vitro* cocaine decreased $GABA_A$ receptor function seen in Chapter 6 is not known.

$GABA_A$ receptors belong to the ligand gated ion channel superfamily. Binding of allosteric modulators to the $GABA_A$ receptor affects the function of the ionophore as seen in Chapters 5 and 6. In addition to modulation by allosteric binding, some of the $GABA_A$ receptor subunits contain amino acid residues that when phosphorylated or dephosphorylated confer additional regulatory influence on the receptor (Pritchett *et al.*, 1989; Leidenheimer *et al.*, 1991a; Swope *et al.*, 1992; Krishek *et al.*, 1994). Phosphorylation sites for PKA and PKC and other kinases exist on $GABA_A$ receptor subunits

(Moss *et al.*, 1992a; Moss *et al.*, 1992b). When these sites were phosphorylated by either PKA or PKC, GABA$_A$ receptor function was reduced (Leidenheimer *et al.*, 1992; Moss *et al.*, 1992b). Thus, phosphorylation of the GABA$_A$ receptor subunits through the activation of PKA or PKC could be a potential mechanism for *in vitro* cocaine to decrease GABA$_A$ receptor function.

The behavioral effects of cocaine is caused by the blockade of DA re-uptake (Ritz *et al.*, 1987). Consequently, D$_1$ and D$_2$ DA receptors are involved in the neurotransmission of the signal. Post-synaptic D$_1$ DA receptors stimulate G$_s$, which leads to the cascade of events involving adenylate cyclase (AC), cAMP and PKA (Cooper *et al.*, 1996). D$_2$ DA receptors are located both pre-synaptically and post-synaptically and stimulate G$_i$, which inhibits the action of AC and DA release (Cooper *et al.*, 1996). Thus, the indirect activation by cocaine of the D$_1$ DA receptor, which leads to the activation of PKA, could be a potential mechanism for decreasing GABA$_A$ receptor function.

In the first part of this dissertation, I have examined the effect of repeated cocaine on striatal GABAergic neurotransmission. In the last chapter, I showed that striatal GABA-stimulated $^{36}Cl^-$ uptake was decreased by *in vitro* cocaine. The mechanism responsible for the acute effect of cocaine may also contribute to the decreased GABAergic neurotransmission after repeated cocaine. Therefore, we examined the effect of *in vitro* cocaine on striatal GABA$_A$ receptor function in order to determine a potential mechanism for these effects. We found that *in vitro* cocaine decreased both striatal GABA-stimulated and striatal muscimol-stimulated $^{36}Cl^-$ uptake only under specific conditions. In addition, a cAMP analog, 8-CPT cAMP, was identified that decreased GABA$_A$ receptor function and may phosphorylate the GABA$_A$ receptor in the same manner as *in vitro* cocaine.

Methods

Animals and Treatment

Male Sprague-Dawley rats were used and housed as described in Chapter 2. Drug naive rats were killed by decapitation and brains were rapidly dissected to obtain striatal and cerebral cortical tissue. Rats were not exposed to *in vivo* cocaine.

GABA$_A$ Receptor $^{36}Cl^-$ Uptake in Brain Microsacs

Brain microsacs from the striatum and cortex were prepared as described in Chapter 2. Briefly, brains were homogenized using a Potter-Elvehjem tissue grinder in ice-cold Ca^{2+} buffer containing protease inhibitors. Homogenates were centrifuged at 900 x *g* for 15 min using a Sorvall SA600 rotor; supernatants were discarded; pellets were resuspended and centrifuged again. The resulting pellets (microsacs) were suspended in Ca^{2+} buffer with protease inhibitors to yield 800-1600 µg of protein per ml.

Next, microsacs were pre-incubated for 5 min at 34°C. The subsequent 3 sec incubation (uptake period) contained both $^{36}Cl^-$ and treatment drugs. Assays with drug pretreatment included the drug in the 5 min pre-incubation period as well as during the uptake period. Uptake was terminated by addition of Ca^{2+} buffer with 100 µM picrotoxin and followed by vacuum filtration onto a glass fiber filter. Two additional washes of Ca^{2+} buffer with picrotoxin were applied onto the filter. The amount of $^{36}Cl^-$ retained on the filter was determined by liquid scintillation. Stimulated uptake was defined as the amount of $^{36}Cl^-$ taken up while agonist was present minus the amount of $^{36}Cl^-$ taken up when agonist was not present. GABA (100 µM) or muscimol (1 –30 µM) were used to stimulate GABA$_A$ receptor $^{36}Cl^-$ uptake. Cocaine (0.1 – 30 µM) was included either only during the uptake period or during both the pre-incubation and uptake periods. A water-soluble forskolin derivative (7-deacetyl-7-O-(N-methylpiperazino-γ-butyryl (compound 3 from (Laurenza *et al.*, 1987)), Calbiochem-Novabiochem Corp, San Diego, CA) was used to activate AC. Analogs of cAMP were used to simulate cAMP activity: 8-(4-chlorophenylthio)-cAMP sodium (8-CPT cAMP (0.1 – 1.0 mM), RBI, Natick, MA), 8-bromoadenosine-3', 5'-cAMP (8-Br cAMP (0.5 – 1.5 mM), RBI, Natick, MA), N^6, 2'-O-dibutyryladenosine 3', 5'-cAMP (Bt_2 cAMP (0.5 – 1.5 mM), Sigma, St. Louis, MO).

Data Analysis

The data are expressed as the mean ± SEM; N = number of subjects. For statistical analysis, ANOVA or repeated measures ANOVA was used on unnormalized $^{36}Cl^-$ uptake data. Bonferroni-Dunn was used for *post-hoc* analysis when appropriate. A p value less than 0.05 was considered to be significant.

Results

Effects of *In Vitro* Cocaine on GABA-Stimulated $^{36}Cl^-$ Uptake in Brain Microsacs

GABA-stimulated $^{36}Cl^-$ uptake was measured in striatal (Figure 7-1) and cerebral cortical (Figure 7-2) microsacs with *in vitro* cocaine either included during the uptake period (3 sec) alone or during both the pre-incubation (5 min) and uptake (3 sec) periods. In the absence of *in vitro* cocaine, striatal and cortical GABA-stimulated $^{36}Cl^-$ uptake were increased by GABA in a dose dependent fashion in both incubation periods. For striatal $^{36}Cl^-$ uptake, *in vitro* cocaine exposure caused a trend towards decreased $GABA_A$ receptor function when included either during pre-incubation and uptake or uptake alone ($p > 0.14$). When this effect of *in vitro* cocaine during the pre-incubation and uptake period is analyzed in isolation, there is a significant effect caused by *in vitro* cocaine on GABA-stimulated $^{36}Cl^-$ uptake [$F(1, 66) = 4.37, p < 0.05$]. *In vitro* cocaine exposure during the uptake period alone did not affect stimulated $^{36}Cl^-$ uptake. For striatal $^{36}Cl^-$ uptake, there was also a significant main effect of incubation periods that was not related to *in vitro* cocaine [$F(1, 126) = 4.05, p < 0.05$]. For cortical $^{36}Cl^-$ uptake, *in vitro* cocaine exposure during either the uptake period alone or both the pre-incubation and uptake period did not significantly decrease GABA-stimulated $^{36}Cl^-$ uptake.

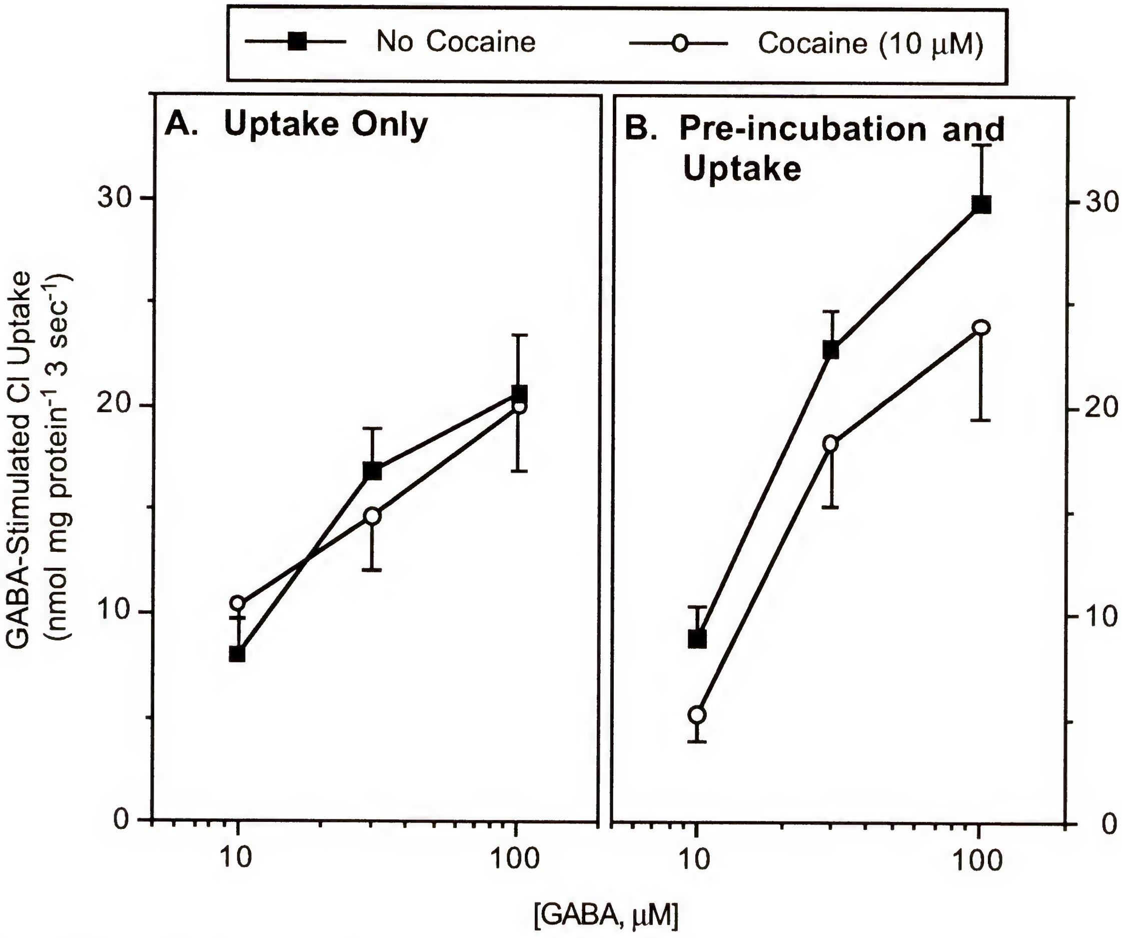

Figure 7-1 The effects of *in vitro* cocaine during the uptake period, or both pre-incubation and uptake periods on GABA-stimulated $^{36}Cl^-$ uptake in striatal microsacs. Exposure of *in vitro* cocaine during both pre-incubation and uptake periods, but not uptake period alone significantly decreases striatal GABA-stimulated $^{36}Cl^-$ uptake. GABA-stimulated (10 – 100 μM) $^{36}Cl^-$ uptake was tested with *in vitro* cocaine (10 μM) during either uptake alone (3 sec) or both pre-incubation (5 min) and uptake (3 sec) periods. A significant effect was observed on GABA-stimulated $^{36}Cl^-$ uptake when in *in vitro* cocaine was included during both the pre-incubation and uptake periods [F(1, 66) = 4.37, $p < 0.05$]. A significant effect was observed between the incubation periods that was not related to *in vitro* cocaine treatment [F(1, 126) = 4.05, $p < 0.05$]. Data shown are the means ± SEM for N = 11 – 13. A major contribution to these data was made by Mr. Paul A. Walker.

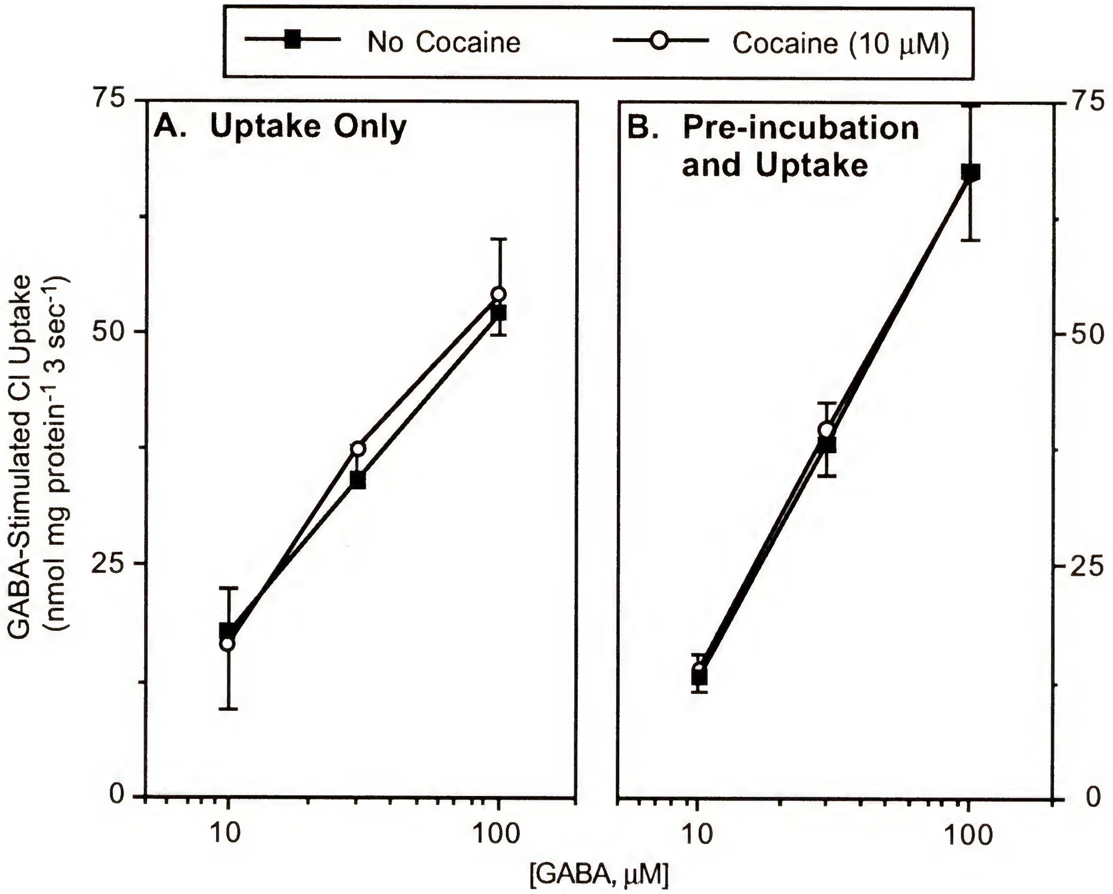

Figure 7-2 The effects of *in vitro* cocaine during uptake period alone, or both pre-incubation and uptake periods on GABA-stimulated$^{36}Cl^-$ uptake in cortical microsacs. The duration of *in vitro* cocaine exposure did not affect cortical $^{36}Cl^-$ uptake. GABA-stimulated (10 – 100 μM) $^{36}Cl^-$ uptake was tested with *in vitro* cocaine (10 μM) during either uptake alone (3 sec) or both pre-incubation (5 min) and uptake (3 sec) periods. Data shown are the means ± SEM for N = 11 – 13. A major contribution to these data was made by Mr. Paul A. Walker.

Cocaine Dose Response on GABA-stimulated and Muscimol-stimulated $^{36}Cl^-$ Uptake

Next, striatal and cortical GABA-stimulated (100 μM) $^{36}Cl^-$ uptake was measured in the presence of different *in vitro* cocaine concentrations (0.1 – 30 μM) during both the pre-incubation and uptake periods (Figure 7-3). *In vitro* cocaine significantly decreased striatal $GABA_A$ receptor function [$F(6, 60) = 2.54$, $p < 0.05$], but not cortical $GABA_A$ receptor function. The decrease in striatal $GABA_A$ receptor function caused by

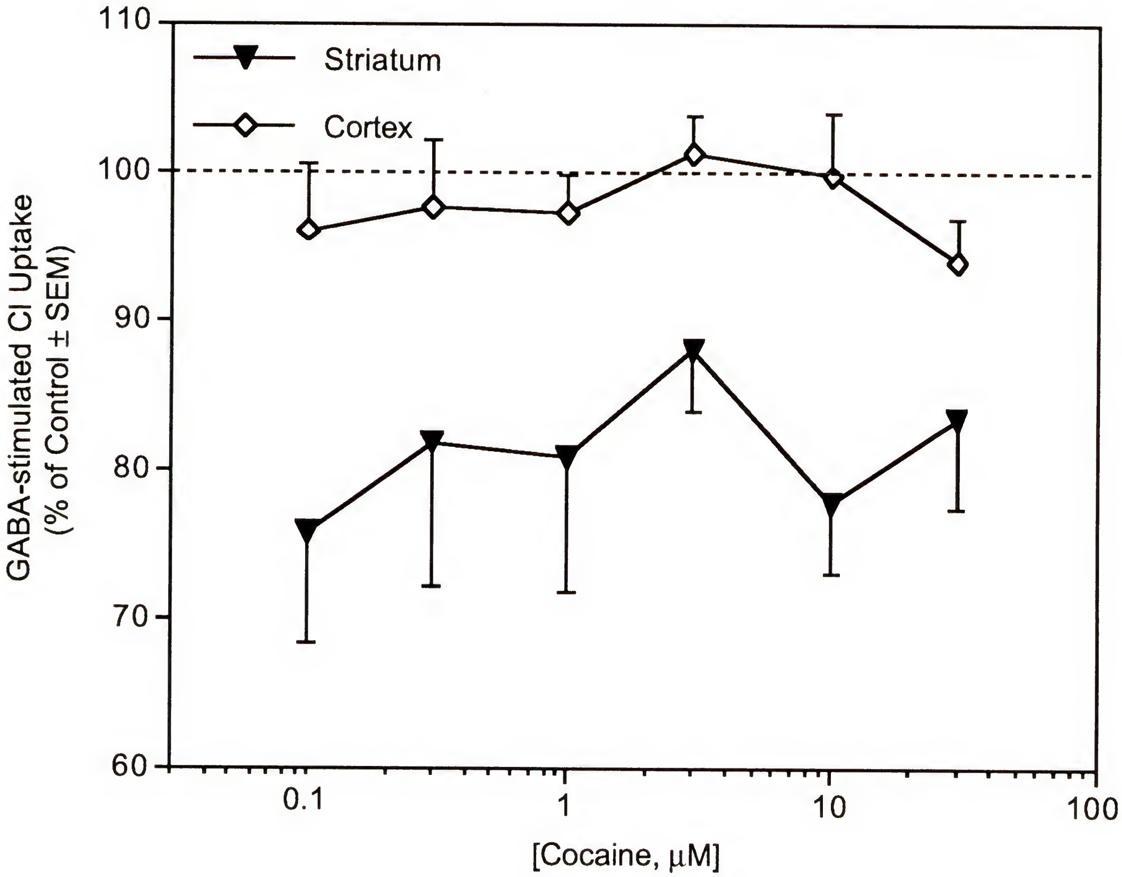

Figure 7-3 Dose response curves for *in vitro* cocaine inhibition of GABA-stimulated $^{36}Cl^-$ uptake in brain microsacs. *In vitro* cocaine decreased striatal stimulated $^{36}Cl^-$ uptake with an approximate maximal decline of 25%. GABA-stimulated $^{36}Cl^-$ uptake was measured with *in vitro* cocaine (0.1 – 30 µM) during both pre-incubation and uptake. The percentage of uptake was calculated by the amount of GABA-stimulated $^{36}Cl^-$ uptake in the presence of *in vitro* cocaine divided by the amount of $^{36}Cl^-$ uptake with 100 µM GABA alone. A significant effect was observed with *in vitro* cocaine on striatal uptake [F(6, 60) = 2.54, $p < 0.05$]. Data shown are the mean percentage ± SEM for N = 6 – 11. A major contribution to these data was made by Mr. Paul A. Walker.

in vitro cocaine ranged from 22.0% to 24.1%, while cortical GABA$_A$ receptor function ranged from 94.1% to 101.3% with increasing concentrations of *in vitro* cocaine.

Striatal muscimol-stimulated $^{36}Cl^-$ uptake was also significantly decreased by *in vitro* cocaine when included during both the pre-incubation and uptake periods [F(3, 21) = 9.52, $p < 0.001$] (Figure 7-4). The range of *in vitro* cocaine tested decreased muscimol-stimulated $^{36}Cl^-$ uptake from 11.9% to 17.7%. In contrast, cortical muscimol-stimulated $^{36}Cl^-$ uptake was not changed by *in vitro* cocaine (Figure 7-5).

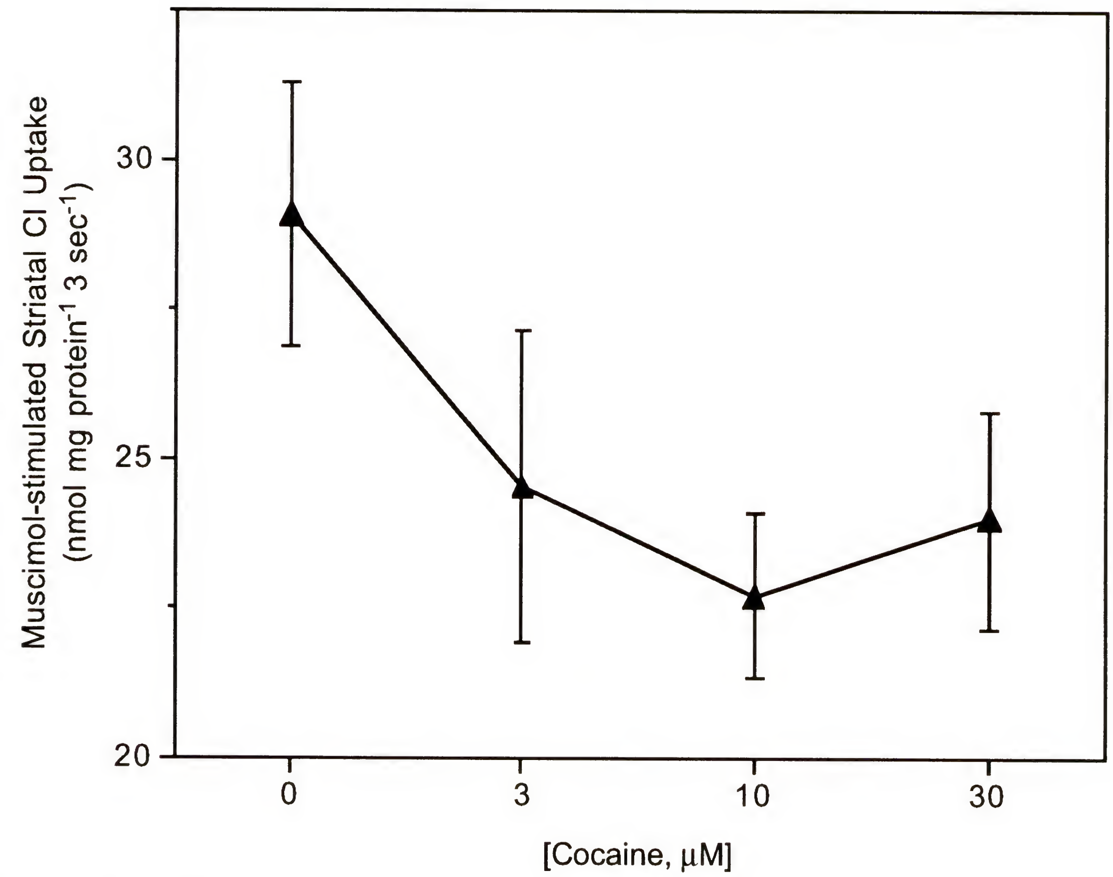

Figure 7-4 The effects of *in vitro* cocaine on muscimol-stimulated striatal $^{36}Cl^-$ uptake. *In vitro* cocaine significantly decreased striatal muscimol stimulated $^{36}Cl^-$ uptake. Muscimol-stimulated (30 μM) $^{36}Cl^-$ uptake was tested with cocaine (3 – 30 μM) during both pre-incubation and uptake periods. A significant effect was observed with *in vitro* cocaine on striatal uptake [F(3, 21) = 9.52, $p < 0.001$]. Data shown are the means ± SEM for N = 8. A major contribution to these data was made by Mr. Paul A. Walker.

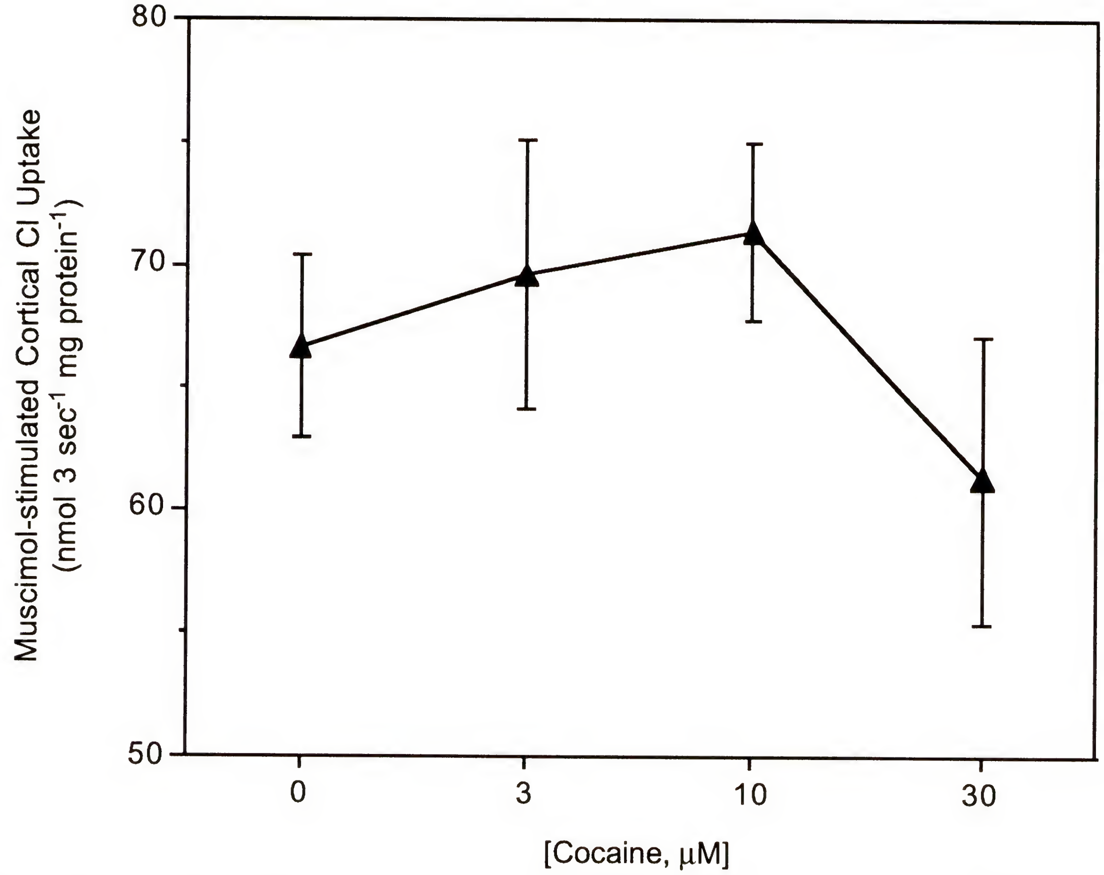

Figure 7-5 The effects of *in vitro* cocaine on muscimol-stimulated cortical $^{36}Cl^-$ uptake. *In vitro* cocaine does not decrease cortical muscimol-stimulated $^{36}Cl^-$ uptake. Muscimol-stimulated (30 μM) $^{36}Cl^-$ uptake was tested with cocaine (3 – 30 μM) during both pre-incubation and uptake periods. Data shown are the means ± SEM for N = 6. A major contribution to these data was made by Mr. Paul A. Walker.

Effects of Forskolin and cAMP Analogs on GABA$_A$ Receptor Function

In order to determine the mechanism for the decrease by *in vitro* cocaine in GABA$_A$ receptor function, it was important to identify second messenger components critical in decreasing GABA$_A$ receptor function. Initially, a forskolin derivative was used to stimulate AC, but this compound did not significantly decrease GABA$_A$ receptor function (data not shown).

Next, cAMP analogs were used to mimic AC activation further downstream of AC. The cAMP analog, 8-CPT cAMP showed the most robust and significant effect on GABA$_A$ receptor function (Figure 7-6). Striatal muscimol-stimulated $^{36}Cl^-$ uptake was significantly decreased by 8-CPT cAMP [F(4, 26) = 25.09, $p < 0.0001$]. Cortical muscimol-stimulated $^{36}Cl^-$ uptake was also significantly decreased by 8-CPT cAMP [F(4, 26) = 33.12, $p < 0.0001$]. The addition of 8-CPT cAMP alone did not have an effect on GABA-independent $^{36}Cl^-$ uptake (data not shown). The other cAMP analogs tested were 8-Br cAMP (Figure 7-7) and Bt_2 cAMP (Figure 7-8). Neither 8-Br cAMP or Bt_2 cAMP had a significant effect on striatal muscimol-stimulated $^{36}Cl^-$ uptake. Cortical muscimol-stimulated $^{36}Cl^-$ uptake was significantly decreased by Bt_2 cAMP [F(3, 16) = 3.93, $p < 0.05$], but not by 8-Br cAMP.

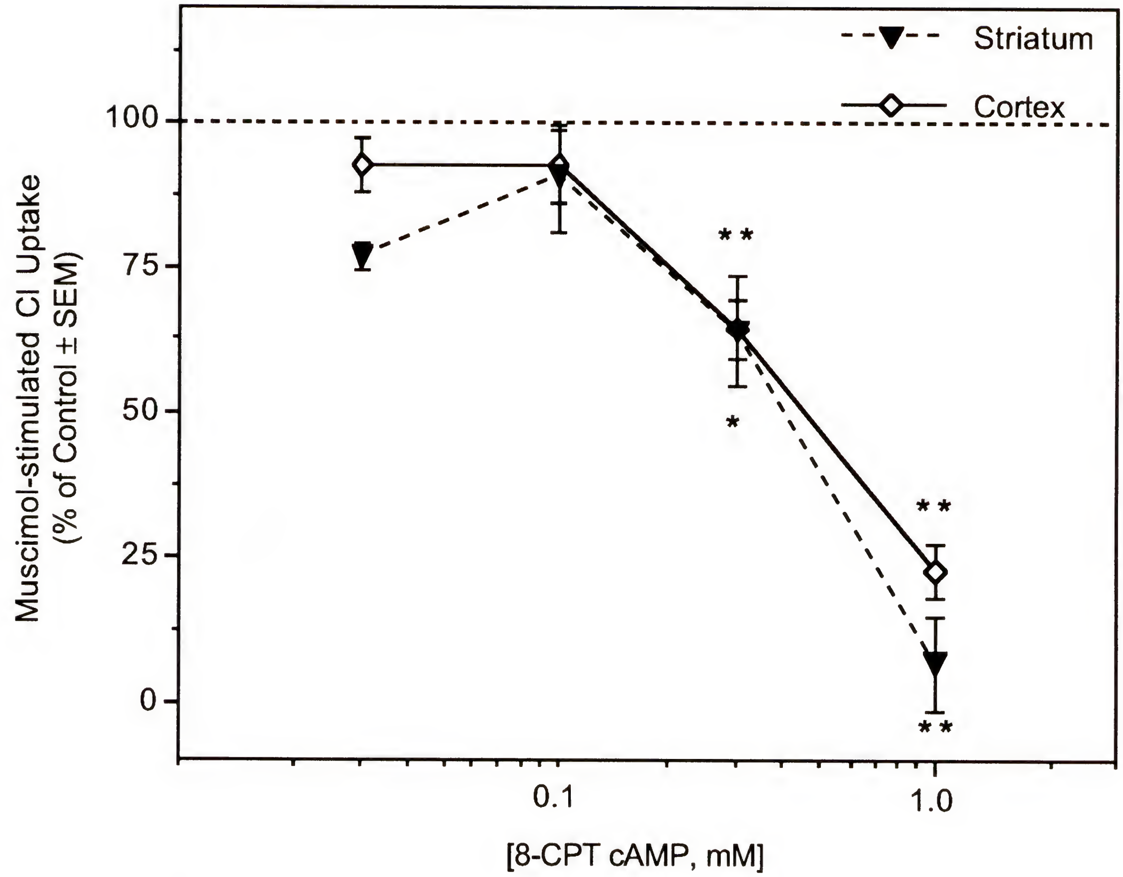

Figure 7-6 The effects of 8-CPT cAMP on muscimol-stimulated $^{36}Cl^-$ uptake in brain microsacs. 8-CPT cAMP significantly decreased muscimol-stimulated $^{36}Cl^-$ uptake both in striatal and cortical microsacs. Muscimol-stimulated (30 μM) $^{36}Cl^-$ uptake was tested with 8-CPT cAMP (0.1 – 1.0 mM) during incubation and uptake periods. The muscimol-stimulated $^{36}Cl^-$ uptake values were 14.79 ± 0.81 nmol 3 sec^{-1} mg $protein^{-1}$ for the striatum and 52.26 ± 2.00 nmol 3 sec^{-1} mg $protein^{-1}$ for the cortex. A significant effect was observed with 8-CPT cAMP on striatal uptake [F(4, 26) = 25.09, $p < 0.0001$] and cortical uptake [F(4, 26) = 33.12, $p < 0.0001$]. Data shown are the mean percentage ± SEM for N = 5 – 7. * $p < 0.05$ vs muscimol control; ** $p < 0.01$ vs muscimol control

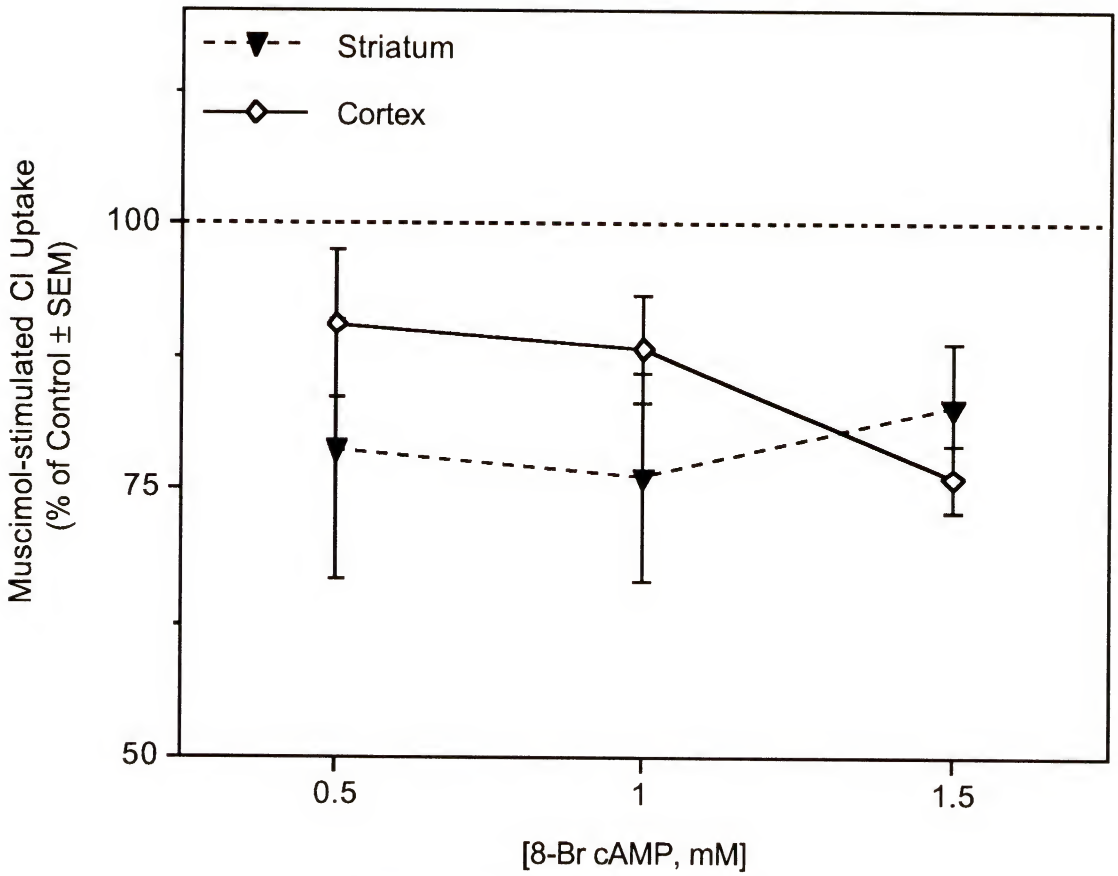

Figure 7-7 The effects of 8-Br cAMP on muscimol-stimulated $^{36}Cl^-$ uptake in brain microsacs. 8-Br cAMP did not significantly decrease muscimol-stimulated $^{36}Cl^-$ uptake in either striatal or cortical microsacs. Muscimol-stimulated (30 μM) $^{36}Cl^-$ uptake was tested with 8-Br cAMP (0.5 – 1.5 mM) during incubation and uptake periods. The muscimol-stimulated $^{36}Cl^-$ uptake values were 20.36 ± 2.60 nmol 3 sec^{-1} mg $protein^{-1}$ for the striatum and 52.81 ± 2.18 nmol 3 sec^{-1} mg $protein^{-1}$ for the cortex. Data shown are the mean percentage ± SEM for N = 5.

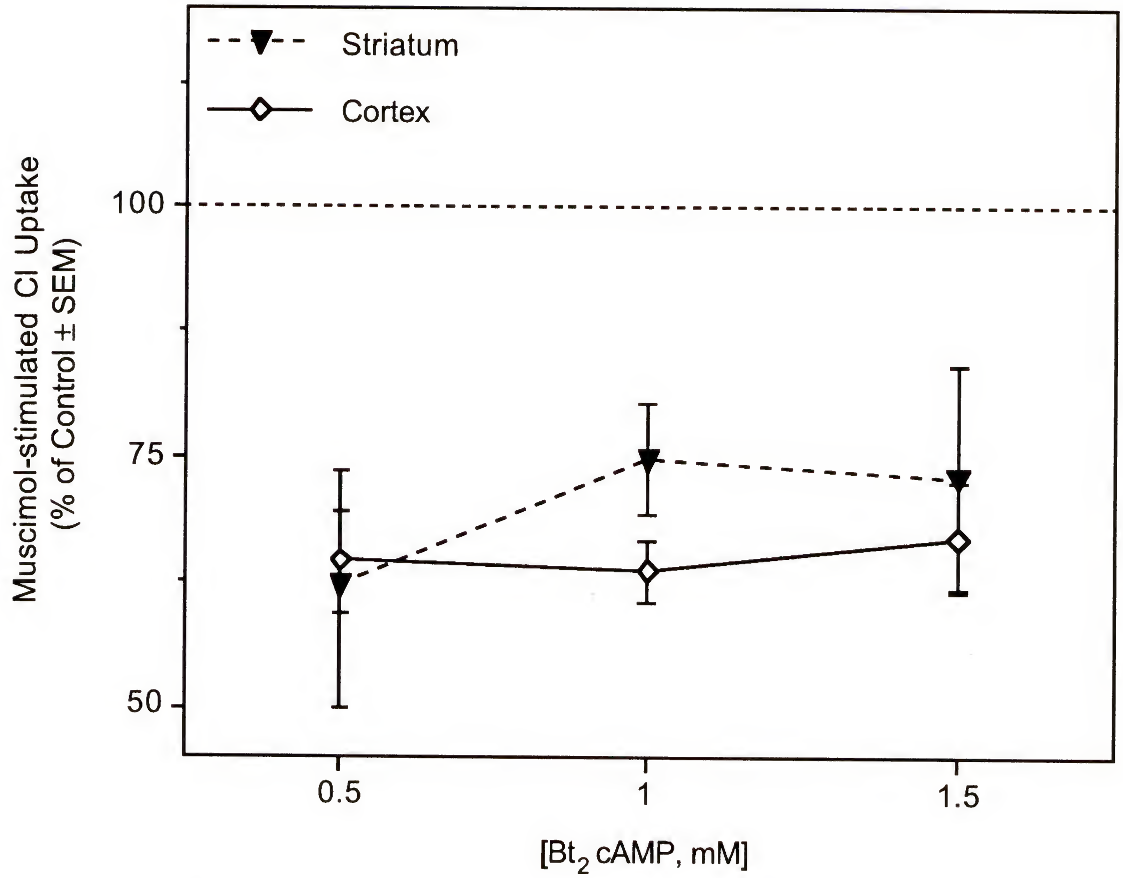

Figure 7-8 The effects of Bt$_2$ cAMP on muscimol-stimulated $^{36}Cl^-$ uptake in brain microsacs. Bt$_2$ cAMP significantly decreased muscimol-stimulated cortical $^{36}Cl^-$ uptake. Muscimol-stimulated (30 μM) $^{36}Cl^-$ uptake was tested with Bt$_2$ cAMP (0.5 – 1.5 mM) during incubation and uptake periods. The muscimol-stimulated $^{36}Cl^-$ uptake values were 24.17 ± 3.24 nmol 3 sec^{-1} mg protein^{-1} for the striatum and 55.91 ± 6.40 nmol 3 sec^{-1} mg protein^{-1} for the cortex. A significant effect was observed in cortical microsacs treated with Bt$_2$ cAMP [F(3, 16) = 3.93, $p < 0.05$]. Data shown are the mean percentage ± SEM for N = 5.

Discussion

As shown previously (Chapter 6), *in vitro* cocaine decreases $^{36}Cl^-$ uptake by striatal GABA$_A$ receptors. Cocaine is generally believed to bind to and block the DAT and prolong DA neurotransmission (Ritz *et al.*, 1987). Thus, the decrease in GABA$_A$ receptor function caused by *in vitro* cocaine was unexpected and contrary to a previous report (Peris, 1996), which may be related to the preparation of microsacs without protease inhibitors in the latter study. In the present study, we found that *in vitro* cocaine decreased both striatal GABA-stimulated and striatal muscimol-stimulated $^{36}Cl^-$ uptake only under specific conditions. I also identified a cAMP analog, 8-CPT cAMP, that reduced striatal $GABA_A$ receptor function and may be acting by a similar mechanism as *in vitro* cocaine.

First, the conditions under which *in vitro* cocaine caused a decrease in striatal $GABA_A$ receptor function were established. Stabilizing the microsac preparation with protease inhibitors (antipain, aprotinin and leupeptin) allowed for the observations that *in vitro* cocaine decreased striatal $GABA_A$ receptor function and also increased the robustness of the $^{36}Cl^-$ uptake assay (J. Peris, unpublished observations). Also critical was the point in the assay when the brain microsac preparation is first exposed to *in vitro* cocaine. *In vitro* cocaine exposure during both the pre-incubation and uptake periods, but not uptake period alone showed decreased striatal $GABA_A$ receptor function (Figure 7-1). This result was obtained with a major contribution by Mr. Paul A. Walker. In future experiments, it would be interesting to determine if extending the pre-incubation period for more than 5 min increases the effect of *in vitro* cocaine on GABA-stimulated $^{36}Cl^-$ uptake. This effect of cocaine exposure could be caused by neurotoxicity. However, the acute or repeated administration (for 3 months) of cocaine (40 mg kg^{-1} i.p.) sufficient to cause sensitization and seizures did not result an observable hippocampal neuronal loss (Goodman and Sloviter, 1993). In addition, it does not seem likely that with the acute *in vitro* cocaine exposure that there is a selective neurotoxic effect on striatal but not cortical membranes.

Next, the dose response effect of *in vitro* cocaine was measured on both striatal and cortical GABA- and muscimol-stimulated $^{36}Cl^-$ uptake. There was a significant decrease in both GABA- and muscimol-stimulated striatal $^{36}Cl^-$ uptake, but not in cortical $^{36}Cl^-$ uptake (Figure 7-3 and Figure 7-4). Again, these results were obtained with a major contribution by Mr. Paul A. Walker. The difference in the effect of *in vitro* cocaine on striatal and cortical $GABA_A$ receptor function may be attributed to the differences in neurotransmitter composition in these regions. The STR is known to contain high concentrations of cocaine binding sites or DATs, while the concentration in the CTX is sparse (Scheffel *et al.*, 1991; Shearman *et al.*, 1996). Hence, the decrease in $GABA_A$ receptor function would then be expected because *in vitro* cocaine could cause a greater increase in dopamine concentration in the STR compared to the CTX. In addition the distribution of the $GABA_A$ receptor subunits in these two regions are also different. The distribution of subunits within the striatum is mainly α_2, α_4, β_3, γ_2 and γ_3, with the other subunits having low expression (Wisden *et al.*, 1992). Cortical subunit composition is more diverse and thus may be differentially affected by *in vitro* cocaine (Wisden *et al.*, 1992). Thus, the effect of *in vitro* cocaine is to decrease striatal $^{36}Cl^-$ uptake, but not cortical $^{36}Cl^-$ uptake.

The concentrations of cocaine (0.1 – 30 μM) used range around the K_i of cocaine (0.7 – 1.51 μM) for the DAT (Ritz *et al.*, 1987; Woodward *et al.*, 1995). In addition, the concentrations of cocaine are within physiologic concentrations measured in the brain (1.9 – 5.8 μM) (Horger *et al.*, 1996). Increasing concentrations of *in vitro* cocaine did not decrease $GABA_A$ receptor function in a dose-dependent manner, rather the decrease occurred in a narrow range both for GABA-stimulated uptake (22.0% – 24.1%) or muscimol-stimulated uptake (11.9% – 17.7%). At cocaine concentrations above 10 μM, it is possible that the effect of *in vitro* cocaine has been already maximized and could also involve the anesthetic effects of cocaine. At lower cocaine concentrations (< 3 μM), *in vitro* cocaine appears to show maximal decreased GABA-stimulated $^{36}Cl^-$ uptake. It is possible that in addition to affecting the DAT, *in vitro* cocaine may also be acting through the 5-HT transporters. In addition, the mechanism for the effect of *in vitro* cocaine may not be through blockade of any neurotransmitter re-uptake transporter. Currently, there is no reported cocaine binding site on the $GABA_A$ receptor, although there are recent reports suggesting an effect of cocaine directly on GABA current (Ye *et al.*, 1997) and a binding pocket on the nicotinic receptor, which may also be on the $GABA_A$ receptor (Francis *et al.*, 1999). However, these mechanisms are preliminary. Future studies should determine what concentrations of *in vitro* cocaine cause no further decreases in $GABA_A$ receptor function to eliminate the possibility of a direct or neurotoxic effect of *in vitro* cocaine.

The mechanism by which *in vitro* cocaine decreased striatal $GABA_A$ receptor function is not known. As stated previously, the primary effect of *in vivo* cocaine is thought to be related to cocaine binding to the DAT and blocking the re-uptake of DA (Ritz *et al.*, 1987), although there is a suggestion of a direct effect of cocaine on $GABA_A$ receptor function (Ye *et al.*, 1997). Thus, I hypothesized that there was an indirect effect of *in vitro* cocaine through other cellular mechanisms. It has previously been observed that phosphorylation of the $GABA_A$ receptor by PKA decreased receptor function in whole cells (Moss *et al.*, 1992b). This decrease was not observed until the cell had been exposed to intracellular cAMP for several minutes (5 – 10 min) (Moss *et al.*, 1992b). In brain synaptoneurosomes, a critical time of 5 min was necessary for cAMP accumulation induced by forskolin to significantly decrease muscimol-stimulated $^{36}Cl^-$ uptake (Heuschneider and Schwartz, 1989). Consequently, a potential mechanism for *in vitro* cocaine was PKA phosphorylation of the $GABA_A$ receptor because of the conditions required to observe the decrease: (1) protease inhibitors, (2) exposure of cocaine during both pre-incubation and uptake periods, and (3) cocaine indirectly affects PKA activation. Thus, decreased $GABA_A$ receptor function by phosphorylation mediated through PKA activation was hypothesized to be the mechanism for *in vitro* cocaine. Therefore, I wanted to test the effects of PKA activators on striatal $GABA_A$ receptor function to determine if a similar decrease to *in vitro* cocaine could be observed.

The decrease by *in vitro* cocaine on $GABA_A$ receptor function may be caused by PKA activation. Consequently, I initially hoped to activate AC with a forskolin derivative that could decrease muscimol-stimulated $^{36}Cl^-$ uptake. Previously shown, this water-soluble forskolin derivative significantly increased cAMP accumulation in cortical synaptoneurosomes, but did not inhibit muscimol-stimulated $^{36}Cl^-$ uptake (Laurenza *et al.*, 1987; Heuschneider and Schwartz, 1989). In confirmation of the previous report in this study, the forskolin derivative did not decrease cortical $GABA_A$ receptor function, and neither did it decrease striatal $GABA_A$ receptor function (data not shown). Although other forskolin derivatives are available, their use could add a potential confound to the assay. These other forskolin compounds are relatively insoluble in water and their potential carriers such as ethanol or DMSO also affect $GABA_A$ receptor function. Thus, I chose to bypass AC activation and examine cAMP analogs that would be downstream of AC activity and activate PKA.

In this study, the effects of three cAMP analogs were measured on both striatal and cortical muscimol-stimulated $^{36}Cl^-$ uptake. As stated previously, the accumulation of cAMP caused by forskolin exposure for 5 min was sufficient to cause decreased cortical synaptoneurosome $^{36}Cl^-$ uptake (Heuschneider and Schwartz, 1989). It has also been shown that PKA is induced by the cAMP analogs used in this study after 5 min of incubation with striatal synaptoneurosomes (Schwartz *et al.*, 1991). The effects of cAMP analogs on synaptoneurosomes have been observed after 10 min of incubation (Heuschneider and Schwartz, 1989). Hence it appears as if the incubation time allows for the cAMP analogs to cross the microsac membrane and activate PKA to phosphorylate proteins. In the current study, the order of efficacy on striatal $^{36}Cl^-$ uptake, 8-CPT cAMP > Bt_2 cAMP = 8-Br cAMP, was similar to a previous report with cortical $^{36}Cl^-$ uptake (Schwartz *et al.*, 1991). 8-CPT cAMP had the largest decrease in both striatal and cortical muscimol-stimulated $^{36}Cl^-$ uptake (Figure 7-6). The decrease in striatal muscimol-stimulated $^{36}Cl^-$ uptake was greater than that found in a previous report on striatal synaptoneurosome muscimol-stimulated $^{36}Cl^-$ uptake (Schwartz *et al.*, 1991). The decrease in cortical muscimol-stimulated $^{36}Cl^-$ uptake caused by 8-CPT cAMP at similar concentrations was also greater than in previous reports (Leidenheimer *et al.*, 1990; Schwartz *et al.*, 1991). The differences that can account for the larger response seen in this study could include different animal species, microsac preparation, 8-CPT cAMP exposure time and muscimol concentration (Leidenheimer *et al.*, 1990; Schwartz *et al.*, 1991). In earlier reports, 8-Br cAMP had been shown to significantly decrease GABA$_A$ receptor function by decreasing hippocampal current and cortical $^{36}Cl^-$ uptake (Harrison and Lambert, 1989; Lambert and Harrison, 1990; Leidenheimer *et al.*, 1990; Schwartz *et al.*, 1991). In this study although neither striatal nor cortical $^{36}Cl^-$ uptake was significantly decreased (Figure 7-7), the decrease in function was approximately within the same range for similar concentrations of 8-Br cAMP as in those earlier reports (Leidenheimer *et al.*, 1990; Schwartz *et al.*, 1991). If the number of experiments were increased and the concentrations of 8-Br cAMP were extended, then it is possible that a significant difference could be observed. Finally, Bt$_2$ cAMP significantly decreased cortical GABA$_A$ receptor function in a range previously reported (Heuschneider and Schwartz, 1989). Again, if the number of experiments were increased and the concentrations of Bt$_2$ cAMP were extended, then it is likely that a significant difference may be observed in striatal uptake. In summary, 8-CPT cAMP caused the largest decrease in muscimol-stimulated $^{36}Cl^-$ uptake.

I hypothesized that phosphorylation of the GABA$_A$ receptor may cause the decrease in receptor function seen with *in vitro* cocaine. If 8-CPT cAMP caused phosphorylation of the GABA$_A$ receptor, then 8-CPT cAMP could be used to examine the effects of phosphorylation of the GABA$_A$ receptor. The mechanism by which 8-CPT cAMP affects GABA$_A$ receptor function has previously been examined (Heuschneider and Schwartz, 1989; Lambert and Harrison, 1990; Leidenheimer *et al.*, 1990; Schwartz *et al.*, 1991). Although 8-CPT cAMP was excellent in stimulating PKA (Schwartz *et al.*, 1991), 8-CPT cAMP has a phosphorylation-independent effect that can decrease GABA$_A$ receptor function (Lambert and Harrison, 1990; Leidenheimer *et al.*, 1990). Thus, using 8-CPT cAMP to phosphorylate the GABA$_A$ receptor could be problematic.

Alternatively, the catalytic subunit of PKA decreased $GABA_A$ receptor function (Porter *et al.*, 1990; Leidenheimer *et al.*, 1991b), but also phosphorylated the $GABA_A$ receptor (Leidenheimer *et al.*, 1991b). Although this preparation is more difficult (the catalytic subunit must first be loaded into the microsacs) this could serve as the model to study the effects of phosphorylation on striatal $GABA_A$ receptor function.

The effect of *in vitro* cocaine was to decrease striatal $GABA_A$ receptor function. Although the variability of decrease makes the examination difficult, it is also possible to inhibit or antagonize the decrease. PKA inhibitors, PKI and H-8, are available, but they are limited by a lack of membrane permeability and inhibition by ATP (Schwartz *et al.*, 1991). It is also possible that PKC may cause the mechanism for the *in vitro* cocaine, but until recently PMA was the only nonspecific inducer of PKC activity (Csukai and Mochly-Rosen, 1999). It would also be interesting to determine the possible role for PKC activity mediating the effect of *in vitro* cocaine. Thus, the pharmacological investigation of the effect of *in vitro* cocaine is restricted by the current tools.

Although we hypothesize that *in vitro* cocaine is activating the PKA second messenger pathway, the mechanism of the initial signal is unknown. In preliminary data, the SCH23390 (D_1 DA receptor antagonist) appears to inhibit the decrease in striatal $GABA_A$ receptor caused by *in vitro* cocaine (J. Peris, personal observation). This suggest that D_1 DA receptor activation may be important for the effects of *in vitro* cocaine. The effects of D_2 DA receptor activation needs to be evaluated in a possible role in regulating the effect of *in vitro* cocaine on $GABA_A$ receptor function. In a recent report, the D_5 DA receptor was shown to downregulate the $GABA_A$ receptor from the cell surface (Man *et al.*, 1999). It is possible that *in vitro* cocaine acting indirectly through the D_5 DA receptor activation could selectively decrease $GABA_A$ receptors. The 5-HT receptor system, which is also affected by cocaine (Rocha *et al.*, 1998), should also be evaluated for its effect on $GABA_A$ receptor function. The possibility of either a cocaine neurotoxic or anesthetic effect should also be considered as the mechanism for *in vitro* cocaine.

In summary, we found that *in vitro* cocaine decreased both striatal GABA-stimulated and muscimol-stimulated $^{36}Cl^-$ uptake only when *in vitro* cocaine was included during the pre-incubation and uptake periods. I hypothesized that this effect was mediated by the PKA second messenger pathway. Attempting to bypass some of the variability of the effect of *in vitro* cocaine, I identified a cAMP analog, 8-CPT cAMP, that reduced striatal $GABA_A$ receptor function. Unfortunately, the use of 8-CPT cAMP may introduce some unique and difficult problems. Further examination of the mechanism of *in vitro* cocaine causing a decrease in $GABA_A$ receptor function should consider receptor systems that potentially regulate PKA phosphorylation pathways.

CHAPTER 8
GENERAL DISCUSSION

In humans, repeated cocaine can lead to the development of behavioral sensitization or reverse tolerance (see Zahniser and Peris, 1992; and Mendelson and Mello, 1996). The rat model is used to study the neurochemical changes associated with the development and maintenance of cocaine sensitization. In rats, cocaine sensitized behavior can be represented by highly stereotypic motions, such as headbobbing, pacing, licking or biting (see Zahniser and Peris, 1992). Cocaine is known to block the DAT (Ritz *et al.*, 1987). Thus, cocaine blocks re-uptake of DA and prolongs the signal transmitted by DA (Ritz *et al.*, 1987). However when cocaine's effects on dopaminergic transmission are carefully examined, the changes are limited and cannot solely account for cocaine sensitization (Kalivas *et al.*, 1993; Kuhar and Pilotte, 1996). This suggests that other neurotransmitter pathways may be involved in mediating behavioral changes associated with repeated cocaine exposure (Pecins-Thompson and Peris, 1993; Karler *et al.*, 1995; Peris, 1996).

The major inhibitory neurotransmitter in the brain is GABA. It has been shown that GABA neurotransmission regulates striatal dopaminergic neurotransmission (Reid *et al.*, 1990a; Reid *et al.*, 1990b; Gruen *et al.*, 1992; Smolders *et al.*, 1995; Morgan and Dewey, 1998). In this laboratory, striatal GABA neurotransmission is hypothesized to be decreased after cocaine sensitization. Previously in this laboratory, $GABA_A$ receptor function and number was shown to be decreased after cocaine sensitization (Pecins-Thompson and Peris, 1993; Peris, 1996). In addition, the absence of the striatal $GABA_A$ receptor $ß_3$ subunit was critical in the development of sensitization (Peris *et al.*, 1998; Resnick *et al.*, 1999). Therefore, a decrease in the tonic inhibitory role of striatal

GABA could contribute to enhanced striatal dopaminergic output caused by repeated cocaine.

The development of behavioral sensitization after repeated cocaine is critical in these experiments. If the rats were not sensitized, then the analysis of the data is confounded. Generally in these experiments, rats receiving repeated cocaine (15 mg kg^{-1}) showed increased behavioral scores with 80% having an increased score on Day 14 compared to Day 1. The increase in behavior was variable. Although human error could be a major factor for this variability, the dose of cocaine could also be a contributing factor. In Chapter 7, I increased the dose of cocaine to 20 mg kg^{-1} and had larger increases in behavioral scores because the range of scores extended to the maximum on the behavioral scale. In addition, cocaine (15 mg kg^{-1}) may be the threshold dose of cocaine to cause sensitization. Increasing the dose of cocaine may not only increase the robustness of cocaine sensitization but may also have greater effects on GABAergic neurotransmission. For example from Chapter 3, the dose of cocaine was critical for the development of cocaine sensitization and changes in endogenous GABA. It would be interesting to determine if increased cocaine concentrations would show further increases in behavior and decreases in endogenous GABA. Thus, optimizing the development of cocaine sensitization by increasing the dose of cocaine could enhance the effect of repeated cocaine on striatal GABAergic neurotransmission.

I found that there was a significant decrease in striatal endogenous GABA release in cocaine sensitized rats (Chapter 3). Hence, the previously reported decrease in striatal $GABA_A$ receptor number is not a compensatory response to increased GABA release. Rather paradoxically, behavioral sensitization causes a decrease in both endogenous GABA release and $GABA_A$ receptors in the striatum, neither of which are compensated in the usual way. These decreases in striatal GABA neurotransmission or inhibitory influences could contribute to enhanced DA neurotransmission and the maintenance of cocaine sensitization. In contrast, repeated cocaine treatment leads to reduced $GABA_B$ receptor function and increased pre-synaptic GABA release in the DLSN (Shoji *et al.*, 1997). However, the changes in the DLSN may be related to the reinforcing effects of repeated cocaine for which drug tolerance is known to develop (Emmett-Oglesby *et al.*, 1993). Thus, changes in striatal endogenous GABA release may be related to the development or maintenance of cocaine sensitization.

Determining the time point at which endogenous GABA release initially declines could indicate the importance of this change to cocaine sensitization. In my experiments, I determined endogenous GABA release at two time points, 24 hours after a single treatment and 24 hours after 14 days of treatment. I found no change in endogenous GABA release after a single dose of cocaine and decreased endogenous GABA release after repeated cocaine. It would be interesting to test periods during the development of cocaine sensitization and periods after cocaine withdrawal. If reduced GABA release occurred with the same developmental time course as cocaine sensitization, this would strongly implicate GABA release as being causal for cocaine sensitization. If reduced GABA release occurred after cocaine sensitization developed, then the persistence of reduced GABA release would indicate the importance to the maintenance of cocaine sensitization. Thus, GABA release during the development of cocaine sensitization and after cocaine withdrawal should be examined.

Currently, vigabatrin (GVG), a GABA transaminase inhibitor, is being developed clinically to prevent drug addictions (Dewey *et al.*, 1998). Already, GVG has been shown to inhibit the increase in DA caused by cocaine (Ashby *et al.*, 1999) through an enhancement in GABA levels (Morgan and Dewey, 1998) and to block cocaine self administration (Kushner *et al.*, 1999). It would also be interesting to determine if GABA levels increased by GVG could prevent cocaine sensitization after repeated cocaine. This could help determine if GABA neurotransmission contributes to cocaine sensitization. Although this would not reveal the cause of cocaine sensitization, prevention of both sensitization and addiction are worthwhile goals.

I hypothesized that decreased GAD activity could be responsible for the decrease in endogenous GABA release. However, I found that GAD activity was not changed by either repeated *in vivo* cocaine or acute, *in vitro* cocaine (Chapter 4). Consequently, I did not determine the mechanism by which endogenous GABA release was decreased. Although I did not find any change in GAD activity, I could not distinguish between the GAD isoenzymes (GAD_{67} or GAD_{65}) or between their activity states (holoenzyme or apoenzyme). Since the isoenzymes of GAD could be unequally affected by repeated cocaine, this could confound the interpretation of my results. Currently, resolving the differences between GAD isozyme activity is difficult because the enzymes are neither easily isolated or simply regulated (Martin and Rimvall, 1993). As of yet, not even the regulation of the enzymes' activity state can be easily measured (Martin and Rimvall, 1993). As a result, the role of GAD in reducing endogenous GABA release will remain an enigma for some time. Thus, it remains possible that synthesis of GABA could still be under the influence of repeated cocaine.

There are many additional places where GABA release could be regulated. Both pre-synaptic release and GABA re-uptake could be used to explain the decrease in GABA. Pre-synaptically endogenous GABA release would be decreased if the packaging or release of GABA synaptic vesicles were changed after cocaine sensitization. Testing a change in synaptic vesicle packaging would be difficult because of the unknowns related to synthesis by GAD and the two pools of GABA (synthetic and metabolic) (Martin and Rimvall, 1993). I hypothesized that differences between endogenous and exogenous GABA release was due to the differing contributions by the synthetic and metabolic GABA pools (Chapter 3). The labeling of the synthetic pool with radioactive GABA precursors and then their consequent release could resolve the contribution of the metabolic pool in the release of endogenous GABA release. Thus, the release of the synthetic GABA pool could be carefully examined after cocaine sensitization. After GABA is released, increased GABA re-uptake could account for the decrease in endogenous GABA. The contribution by increased re-uptake to the decrease in endogenous GABA needs to be evaluated to convincingly assert that there is no effect of re-uptake.

The reported changes in striatal $GABA_A$ receptor number after repeated cocaine are dissimilar. For instance, there has been reports of an increase in [^{3}H]-Ro 15-1788 binding and a decrease in [^{36}S]-TBPS binding (Goeders, 1991; Pecins-Thompson and Peris, 1993). My results do not resolve this controversy. We hypothesized that the $GABA_A$ receptor may be uncoupled from the allosteric modulatory site after cocaine sensitization. I showed that there is no change in binding to either the GABA site or BZD site after repeated cocaine (Chapter 5). In addition, modulated binding at these sites was also unchanged. It is possible that repeated cocaine affects the functional affinity of the modulatory site as opposed to its number. Testing this hypothesis would require the use of more concentrations of the allosteric ligand.

The time course of the changes in BZD binding also needs to be considered. The initial report measured BZD binding after repeated cocaine was withdrawn for 20 min, 2 days and 14 days (Goeders, 1991). BZD binding was only significantly decreased 20 min after withdrawal. At 2 or 14 days after withdrawal, BZD binding returned to baseline. In my study, I examined BZD binding 24 hr after withdrawal and found no difference in BZD binding between saline and cocaine treated rats (Chapter 5). There are several possible explanation for these results. First, cocaine remaining in the brain affects BZD binding. The possibility that *in vivo* cocaine contributed to the decrease seen at 20 min after withdrawal was eliminated by an experiment that showed 20 min of acute *in vivo* cocaine did not affect BZD binding (Goeders, 1991). Second, BZD binding after cocaine withdrawal is not an indicator for cocaine sensitization. Cocaine sensitization is known to be persistent. Hence, the ability of the $GABA_A$ receptor to quickly regain BZD binding after cocaine withdrawal would not be a persistent change caused by repeated cocaine. This result emphasizes the importance of determining both the initiation and duration of changes that occur after cocaine sensitization.

Changes in functional modulation of striatal GABA$_A$ receptor binding after repeated cocaine could contribute to cocaine sensitization. Although I found that there was no change in functional modulation of binding to striatal GABA$_A$ receptor 24 hr after withdrawal (Chapter 5), others have found in animals chronically treated with ethanol or BZD that the main GABA site was uncoupled from the BZD site (Gallager *et al.*, 1984; Mele *et al.*, 1984; Tietz *et al.*, 1989). In addition, the shift in BZD affinity was changed after chronic drug treatment (Klein *et al.*, 1995). In my experiments, I did not measure either BZD affinity or shifts in BZD affinity after cocaine sensitization since I used a single concentration of BZD. Thus, the ability of GABA to shift the affinity of BZD binding needs to be examined after cocaine sensitization.

In addition to functional modulation of binding, GABA$_A$ receptor $^{36}Cl^-$ uptake can be modulated by allosteric ligands. In this study, I found that cocaine sensitization did not result in a change in allosterically modulated striatal GABA$_A$ receptor $^{36}Cl^-$ uptake 24 hr after withdrawal (Chapter 6). Previously when GABA$_A$ receptor $^{36}Cl^-$ uptake was measured after chronic ethanol or BZD, the BZD site was uncoupled from the GABA site only for a short period after withdrawal (0 – 6 hr) (Buck and Harris, 1990; Li *et al.*, 1993). In this study, it is possible that the receptor may be initially decoupled, then recoupled sometime during the 24 hr withdrawal period. Because decoupling would be potentially brief and decreased BZD binding was not persistent (Chapter 5; Goeders, 1991), it is worthwhile to mention again that the allosteric BZD site may play a less significant role in the maintenance of cocaine sensitization.

Cocaine is generally believed to bind to and block the DAT thereby prolonging DA neurotransmission (Ritz *et al.*, 1987). Consequently, the decrease in GABA$_A$ receptor function caused by *in vitro* cocaine was unexpected (Chapter 6). In Chapter 7, I expanded the dose of *in vitro* cocaine used and found that it decreased both GABA-stimulated and muscimol-stimulated striatal $^{36}Cl^-$ uptake, but not cortical $^{36}Cl^-$ uptake. The decrease in $GABA_A$ receptor function was only observed when striatal microsacs were pre-incubated with *in vitro* cocaine. The mechanism by which *in vitro* cocaine decreased $GABA_A$ receptor function is not known.

Currently, there is no reported cocaine binding site on the $GABA_A$ receptor, although there are reports suggesting a direct effect of cocaine on the $GABA_A$ receptor. Cocaine has been shown to decrease GABA currents, but this may be due to an anesthetic effect because the IC_{50} for cocaine was high (0.13 – 1.2 mM) at all concentrations of GABA treatment (2 – 100 μM) (Ye *et al.*, 1997; Ye *et al.*, 1999). Indirectly, cocaine also inhibited nicotinic acetylcholine receptors on sites that may have some homology with the $GABA_A$ receptor subunits (Francis *et al.*, 1999). Hence, direct interactions with cocaine and the $GABA_A$ receptor remain a possibility.

However, I hypothesized that there was an indirect effect of *in vitro* cocaine through another cellular mechanism. It has previously been observed that phosphorylation of the $GABA_A$ receptor by PKA decreased $GABA_A$ receptor function (Moss *et al.*, 1992). This decrease was not observed until the cell had been exposed to intracellular cAMP for several minutes (5 – 10 min) (Moss *et al.*, 1992). In brain synaptoneurosomes, a critical time of 5 min was necessary for cAMP accumulation induced by forskolin to significantly decrease muscimol-stimulated $^{36}Cl^-$ uptake (Heuschneider and Schwartz, 1989). Thus, the PKA second messenger pathway seemed to be a likely candidate as a potential mechanism for the decrease in $^{36}Cl^-$ uptake by *in vitro* cocaine because of the conditions required to observe the decrease: (1) protease inhibitors and (2) exposure of cocaine during both pre-incubation and uptake periods. From these results, I hypothesized that PKA was involved in the phosphorylation of the $GABA_A$ receptor and consequently decreased receptor function.

The activation of the PKA second messenger pathway by *in vitro* cocaine could the mechanism for the decrease in striatal $GABA_A$ receptor $^{36}Cl^-$ uptake. If D_1 DA or D_5

DA receptors were indirectly stimulated through *in vitro* cocaine elevation of DA levels, then the resulting activation of the PKA second messenger cascade could eventually result in the phosphorylation of the $GABA_A$ receptor. I examined the effects of intermediates of the PKA second messenger cascade on $GABA_A$ receptor function. In this study, I found that 8-CPT cAMP significantly decreased striatal $GABA_A$ receptor $^{36}Cl^-$ uptake (Chapter 7). However, the mechanism of 8-CPT cAMP may not be mediated by the phosphorylation of the $GABA_A$ receptor as I have hypothesized (Lambert and Harrison, 1990; Leidenheimer *et al.*, 1990). Alternatively, the effects of *in vitro* cocaine could be inhibited by PKA inhibitors. Currently, the number of PKA inhibitors that could be used have limitations, but there are others such as Rp cAMPS that have fewer limitations. Thus, the importance of phosphorylation of the $GABA_A$ receptor after *in vitro* cocaine remains to be determined.

In addition to the regulation of the $GABA_A$ receptor by phosphorylation, there exist several additional mechanisms that could decrease $GABA_A$ receptor function. One mechanism to decrease receptor function would be to reduce the amount of $GABA_A$ receptors. A recent report suggests that D_5 DA receptors regulate the expression of cell surface $GABA_A$ receptors (Man *et al.*, 1999). This could be a novel way that increases of DA by repeated cocaine could regulate the expression and function of striatal $GABA_A$ receptors. In addition, as this laboratory has shown, the regulation of individual $GABA_A$ receptor subunits could alter the expression of cocaine sensitization. It was determined that the expression of the $GABA_A$ receptor β_3 subunit is critical for the development and expression of cocaine sensitization (Peris *et al.*, 1998; Resnick *et al.*, 1999). Consequently, the regulation of $GABA_A$ receptor subunit expression could control not only the expression, but also the function of the $GABA_A$ receptor. Thus, additional mechanisms that would decrease $GABA_A$ receptor function after *in vitro* cocaine exist and should be examined.

In this dissertation, I found that GABA release is decreased after cocaine sensitization (Chapter 3). The decrease in GABA may be related to the dose of cocaine, because only the sensitizing dose of cocaine decreased release. When GAD activity was measured after cocaine sensitization, I found that there was no difference between repeated saline and cocaine treated rats (Chapter 4). In addition, acute *in vitro* cocaine did not have an effect on GAD activity. Next, post-synaptic GABA neurotransmission was examined. I found that neither allosteric modulation of $GABA_A$ receptor binding nor allosteric modulation of $GABA_A$ receptor $^{36}Cl^-$ uptake was changed by repeated cocaine (Chapters 5 and 6). I was able to identify a decrease in $GABA_A$ receptor function by *in vitro* cocaine that was not related to repeated cocaine (Chapter 6). We examined this effect of *in vitro* cocaine on $GABA_A$ receptor function and found that the decrease was restricted in striatal, but not cortical $GABA_A$ receptor function (Chapter 7). I hypothesized that this effect was caused by phosphorylation of the $GABA_A$ receptor by PKA. I identified a cAMP analog, 8-CPT cAMP, that decreased $GABA_A$ receptor function, but the mechanism for the effect may not be related to the phosphorylation of the $GABA_A$ receptor. Thus, the only measure of striatal GABAergic neurotransmission decreased after repeated cocaine was endogenous GABA release. We also observed a novel effect of *in vitro* cocaine that may be related to the phosphorylation state of the $GABA_A$ receptor. Thus, the decline in pre-synaptic GABAergic neurotransmission could enhance the effect of cocaine stimulated DA neurotransmission and contribute to cocaine sensitization.

Finally, the decline in GABAergic neurotransmission after repeated cocaine may be important in a clinical situation. In addition to behavioral sensitization, repeated cocaine can both induce seizures and increase seizure susceptibility, but this may be related to the anesthetic properties of cocaine (Marley and Gallager, 1989; Goodman and Sloviter, 1993). However, the role of GABA neurotransmission in cocaine seizure susceptibility is being investigated (Abel and Kirages, 1995; Olinger *et al.*, 1999). In addition, diazepam, a BZD, has been clinically used to treat cocaine-induced seizures (Das, 1993). Thus, decreased efficacy of GABAergic neurotransmission may also contribute to cocaine seizure susceptibility. Consequently, the pharmacological treatment may involve first understanding the changes occurring to GABAergic neurotransmission after repeated cocaine.

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BIOGRAPHICAL SKETCH

Bruce John Jung was born in Los Angeles and raised in the now - infamous suburb of Monterey Park. Although he was attracted to studying dinosaurs (paleontology) at an early age — before Jurassic Park and Barney — by the time he had graduated from Alhambra High School, he figured that he had a knack for the biological sciences (luckily for founders of Yahoo! and Netscape, because computer science was his other calling). He enrolled at the University of California at Los Angeles in the hopes of becoming a well-paid, brilliant scientist. Alas, the medical school weeder classes he took with his friends destroyed his grade point average, but not his scientific curiosity. With his supposed well-rounded bachelor's degree, he found a job doing basic cell biology research, but at the rival institution to his alma mater. Bruce learned a lot about science during these years, but knew that he must get a more rigorous scientific training if he really wanted to go anywhere.

After a relaxing but financially unrewarding trip to Las Vegas, Bruce found a (lucky) letter beckoning him to the University of Florida in Gainesville (even before Chancellor/President Charles Young received his). Bruce obtained more than he bargained for. In addition to receiving a rigorous scientific training, he finally enjoyed a college football program that was a consistent winner (Go Gators!!!). Obviously, Bruce has learned a lot about $GABA_A$ receptors and cocaine, but he does not yet know how his knowledge will contribute to the world. In the near future, you will probably find Bruce readily enjoying his copy of The Wall Street Journal. In the distant future, who knows, you may even get to read about him in it.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joanna Peris

Joanna Peris, Chair
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James W. Simpkins
Professor of Pharmacodynamics

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2000

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